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(54) Title: INTERMEDIATES AND ENZYMES OF THE NON-MEVALONATE ISOPRENOID PATHWAY

(57) Abstract: The invention provides a protein in a form that is functional for the enzymatic conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-but enyl 4-diphosphate notably in its (E)-form of the non-mevalonate biosynthetic pathway to isoprenoids. The invention also provides a protein in a form that is functional for the enzymatic conversion of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably in its (E)-form, to isopentenyl diphosphate and/or dimethylallyl diphosphate. Further, screening methods for inhibitors of these proteins are provided. Further, 1-hydroxy-2-methyl-2-but enyl 4-diphosphate is provided and chemical and enzymatic methods of its preparation.

Intermediates and enzymes of the non-mevalonate isoprenoid pathway

Field of the invention

The present invention relates to cells, cell cultures or organisms or parts thereof for the efficient formation of a biosynthetic product or intermediate or enzyme of a 1-deoxy-D-xylulose 5-phosphate-dependent biosynthetic pathway. Further, the invention relates to vectors for producing them. Further, the invention relates to their use for the formation or production of intermediates or products or enzymes of said biosynthetic pathway as well as to enzymes and intermediates. Further, the invention relates to the screening for inhibitors or enzymes for said biosynthetic pathway.

Background of the invention

The system of biosynthetic pathways in any organism is highly streamlined, whereby a few central trunk pathways branch into a great number of peripheral pathways. The central trunk pathways involve starting materials which are highly integrated. Therefore, central or trunk pathways are highly regulated. At the same time they are crucial for any attempts to interfere with the metabolism of any organism either by an inhibitor or by metabolic engineering.

The isoprenoid pathways are a prime example for this metabolic organisation. They are very long and highly branched, leading to some 30,000 isoprenoid or terpenoid compounds. They all seem to derive from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). They are produced by two alternative trunk pathways (reviewed in Eisenreich et al., 2001).

By the classical research of Bloch, Cornforth, Lynen and co-workers, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) have become established as key intermediates in the biosynthesis of isoprenoids via mevalonate. However, many bacteria, plastids of all plants, and the protozoon *Plasmodium falciparum* synthesize IPP and DMAPP by an alternative pathway via 1-deoxy-D-xylulose 5-phosphate. The discovery of the pathway was mainly based on the incorporation of isotope-labelled 1-deoxy-D-xylulose into the isoprenoid side chain of menaquinones from *Escherichia coli* (Arigoni and Schwarz, 1999).

This mevalonate-independent pathway has so far only been partially explored (Fig. 1). For a better understanding of these aspects of the invention, the pathway shall be briefly explained.

It can be divided into three segments:

In a first pathway segment shown in Fig. 1, pyruvate (1) is condensed with glyceraldehyde 3-phosphate (2) to 1-deoxy-D-xylulose 5-phosphate (DXP) (3). Subsequently, DXP is converted into 2C-methyl-D-erythritol 4-phosphate (MEP) (4) by a two-step reaction comprising a rearrangement and a reduction. This establishes the 5-carbon isoprenoid skeleton.

In the subsequent segment of the mevalonate-independent pathway (Fig. 1), MEP (4) is first condensed with CTP to 4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) (5) by 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (PCT/EP00/07548). CDP-ME (5) is subsequently ATP-dependent phosphorylated by 4-diphosphocytidyl-2C-methyl-D-erythritol kinase yielding 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDP-MEP) (6). The intermediate is subsequently converted into 2C-methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP) (7) by 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (PCT/EP00/07548). These three enzymatic steps form a biosynthetic unit which activates the isoprenoid C₅-skeleton for the third pathway segment (Rohdich *et al.*, 1999; Lüttgen *et al.*, 2000; Herz *et al.*, 2000).

Bioinformatic studies (German Patent Application 10027821.3), as well as studies with mutants of *Synechocystis* sp. (Cunningham *et al.*, 2000) and *Escherichia coli* (Campos *et al.*, 2001; Altincicek *et al.*, 2001) demonstrate the involvement of *lytB* and *gcpE* genes in the isoprenoid pathway. However, the function and the reaction catalyzed by the corresponding gene products are still unknown.

Recently, a kinase (XylB) has been described that catalyzes the conversion of 1-deoxy-D-xylulose into 1-deoxy-D-xylulose 5-phosphate at high rates (Wungsintaweekul *et al.*, 2000). Genes and enzymes participating in further downstream reactions have been described. However, the gene functions, the intermediates, and the mechanisms leading to the products are still unknown.

For numerous pathogenic eubacteria as well as for the malaria parasite *P. falciparum*, the enzymes involved in the non-mevalonate pathway are essential. The intermediates of the mevalonate-independent pathway cannot be assimilated from the environment by pathogenic eubacteria and *P. falciparum*. The enzymes of the alternative isoprenoid pathway do not occur in mammalia which synthesize their isoprenoids and terpenoids exclusively via the mevalonate pathway. Moreover, the idiosyncratic nature of the reactions in this pathway reduces the risk of cross-inhibitions with other, notably mammalian enzymes.

Therefore, enzymes of the alternative isoprenoid pathway seem to be specially suited as

targets for novel agents against pathogenic microorganisms and herbicides. The elucidation of unknown steps and the identification of these targets, e.g. genes and cognate enzymes of these pathways is obligatory for this purpose.

A further source of interest in the non-mevalonate pathway derives from the fact certain pathogens like *Mycobacteria*, *Plasmodia*, *Escherichia* etc. use this pathway to activate $\gamma\delta$ T cells (Fournié and Bonneville, 1996). Therefore, $\gamma\delta$ T cells likely act as a first line of defense against infections by such pathogens. Intermediates of the non-mevalonate pathway have been suggested to be responsible for $\gamma\delta$ T cell activation (Jomaa *et al.*, 1999). Recently, it was shown that *E. coli* strains lost the ability to stimulate $\gamma\delta$ T cells when the *dxr* or the *gcpE* gene was knocked out (Altincicek *et al.*, 2001).

Moreover, there is a great biotechnological interest in these pathways, since they lead to valuable vitamins and isoprenoid or terpenoid products.

Previous attempts to approach these goals have been hampered by the low rate of biosynthesis along these pathways in wild-type cells studied so far.

Summary of the invention

It is an object of the invention to provide enzymes and nucleic acids coding for said enzymes as well as intermediates for the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to isopentenyl diphosphate and/or dimethylallyl diphosphate.

It has surprisingly been found that the intermediate in the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to isopentenyl diphosphate and/or dimethylallyl diphosphate is 1-hydroxy-2-methyl-2-but enyl 4-diphosphate. This intermediate is formed by an enzyme encoded by *gcpE* as designated in the *E. coli* genome. It has further been found that this enzyme prefers as reductant NADH or NADPH. Further, it has been found that it is promoted by Co^{2+} .

The above intermediate is converted to isopentenyl diphosphate and/or dimethylallyl diphosphate by an enzyme encoded by *lytB* as designated in the *E. coli* genome. The latter enzyme prefers as reductant NADH or NADPH and FAD as mediator. Further it can be promoted by ions of a metal selected from manganese, iron, cobalt, nickel.

With these findings, the third segment of the trunk non-mevalonate pathway is now

established. The key to these findings is the intermediate 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably in its *E*-form. This establishes the unifying principle of the invention for reactions to and from this intermediate.

Further, it is an object of the invention to provide cells, cell cultures, organisms or parts thereof for the efficient biosynthesis of isoprenoid products or intermediates of the non-mevalonate biosynthetic pathway dependent on 1-deoxy-D-xylulose 5-phosphate production from 1-deoxy-D-xylulose and/or glucose.

The present invention produces a novel *in vivo* system which can be used for the structure elucidation of unknown intermediates and the assignment of biological functions of putative genes or cognate enzymes in the alternative isoprenoid biosynthetic pathway. As an example, the functional assignment of the *gcpE* gene (now designated as *ispG*) and of the *lytB* gene (now designated *ispH*) in the mevalonate-independent pathway of isoprenoid biosynthesis is achieved.

More specifically, said *in vivo* system consists of recombinant *E. coli* strains harbouring vector construct(s) carrying and expressing genes for D-xylulokinase (*xyI**B*), and genes of further downstream steps of terpenoid biosynthesis, such as *dxs*, *dxr*, and/or *ispD*, and/or *ispE*, and/or *ispF*, and/or *gcpE*, and/or *lytB* from *E. coli*, and/or a carotenoid gene cluster from *Erwinia uredovora*.

In one aspect of the invention, the genetically modified strains can be fed with 1-deoxy-D-xylulose, notably with isotope-labelled 1-deoxy-D-xylulose, which is converted at high rates into the common intermediate of the mevalonate-independent terpenoid pathway, 1-deoxy-D-xylulose 5-phosphate, and into further intermediates of said pathway, like 2C-methyl-D-erythritol 4-phosphate, 4-diphosphocytidyl-2C-methyl-D-erythritol, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate, 2C-methyl-D-erythritol 2,4-cyclodiphosphate, 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, isopentenyl diphosphate, and dimethylallyl diphosphate.

Further, feeding with glucose or an intermediate of glycolysis for conversion into said further intermediates of said pathway may be performed.

Said systems are useful for the structure elucidation of hitherto elusive intermediates in the

biosynthetic pathways, for *in vivo* screening of novel antibiotics, antimalarials, and herbicides, and as a platform for the bioconversion of exogenous 1-deoxy-D-xylulose and/or glucose into intermediates and products of the non-mevalonate pathway of terpenoid biosynthesis.

Said systems can also be used for screening chemical libraries for potential herbicides, and/or antimalarials, and/or antimicrobial substances by detecting and measuring the amount of certain intermediates formed *in vivo* in the presence or absence of potential inhibitors of the gene products of mevalonate-independent isoprenoid pathway genes, namely *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *gcpE*, and *lytB*.

Said system can further be used for the production of higher isoprenoids (e.g. isoprenoids having 10, 15, 20, 30 or 40 carbon atoms) such as carotene, α -tocopherole or vitamins by boosting the biosynthesis of isopentenyl diphosphate and/or dimethylallyl diphosphate via the non-mevalonate pathway, e.g. by using glucose as feeding material. Further feeding materials which may be used are intermediates or products of glycolysis like glyceraldehyde 3-phosphate or pyruvate.

Further, this invention provides novel compounds of formula I (see below), notably 1-hydroxy-2-methyl-2-butenyl 4-diphosphate as well as enzymatic and chemical methods for preparing said compounds. As demonstrated herein, (*E*)-1-hydroxy-2-methyl-2-butenyl 4-diphosphate is produced from 2C-methyl-D-erythritol 2,4-cyclodiphosphate by the *gcpE* gene product.

It is further demonstrated herein that (*E*)-1-hydroxy-2-methyl-2-butenyl 4-diphosphate is converted to dimethylallyl diphosphate and isopentenyl diphosphate by the *lytB* gene product.

Short description of the figures and annexes

Figure 1: Biosynthesis of both isoprenoid precursors, isopentenyl pyrophosphate and dimethylallyl pyrophosphate via the mevalonate-independent pathway.

Figure 2: Scheme of an *Escherichia coli* *in vivo* system for generating optionally isotopically labelled intermediates of biosynthetic pathways such as the mevalonate-independent isoprenoid biosynthesis, and for the production of higher terpenoids such as carotenoids.

Figure 3: ^1H NMR spectra in D_2O (pH 6) obtained according to Example 25. * indicates impurities.

Figure 4: Preparation of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate according to Example 24. Reagents and conditions were as follows: (a) DHP, PPTS, 25 °C (2.5 h); (b) $\text{Ph}_3\text{PCHCO}_2\text{Et}$, toluene, reflux (39 h); (c) (1) DIBAH, CH_2Cl_2 , -78 °C (3 h), (2) 1 M $\text{NaOH}/\text{H}_2\text{O}$; (d) $p\text{-TsCl}$, DMAP, CH_2Cl_2 , 25 °C (1 h); (e) $((\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2)_4\text{N})_3\text{HP}_2\text{O}_7$, MeCN, 25 °C (2 h); (f), $\text{HCl}/\text{H}_2\text{O}$ pH 1, 25 °C (7 min).

Figure 5: The reaction catalyzed by the *ispH* (formerly *lytB*) gene product.

Figure 6: The reaction catalyzed by the *ispG* (formerly *gcpE*) gene product.

Figure 7: Chemical preparation of 3-formyl-but-2-enyl 1-diphosphate (see example 42).

Annex A: DNA sequence of the vector construct pBSxylBdxr.

Annex B: DNA sequence of the vector construct pBSxylBdxrispD.

Annex C: DNA sequence of the vector construct pBScyclo.

Annex D: DNA sequence of the vector construct pACYCgcpE.

Annex E: DNA sequence of the vector construct pBScaro14.

Annex F: DNA sequence of the vector construct pACYCcaro14.

Annex G: DNA sequence and corresponding amino acid sequence of the *ispG* (formerly *gcpE*) gene from *Escherichia coli*.

Annex H: DNA sequence of the vector construct pBScyclogcpE.

Annex I: DNA sequence of the vector construct pACYClytBgcpE.

Annex J: DNA and corresponding amino acid sequence of the *ispH* (formerly *lytB*) gene from *Escherichia coli*.

Annex K: DNA sequence of the vector construct pBScyclogcpElytB2.

Annex L: DNA and corresponding amino acid sequence of the *ispG* gene (fragment) from *Arabidopsis thaliana*.

Annex M: DNA and corresponding amino acid sequence of the *ispG* (formrily *gcpE*) gene of *Arabidopsis thaliana*.

Annex N: cDNA sequence of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (IspH) from *Arabidopsis thaliana*

Detailed description of the invention

1-Deoxy-D-xylulose 5-phosphate is a common intermediate in the alternative terpenoid pathway via 2C-methyl-D-erythritol 4-phosphate. This latter pathway is operative in bacteria, certain protozoa and most significantly also in the plastids of plants, where it is in charge of the biosynthesis of a great many valuable terpenoid products, like natural rubber, carotenoids, menthol, menthone, camphor or paclitaxel. The alternative terpenoid pathway is now intensely studied. But so far only the initial steps from glyceraldehyde 3-phosphate and pyruvate via 1-deoxy-D-xylulose 5-phosphate and 2C-methyl-D-erythritol 4-phosphate, 4-diphosphocytidyl-2C-methyl-D-erythritol, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate and 2C-methyl-D-erythritol 2,4-cyclodiphosphate (Fig. 1) have been elucidated.

The intermediate 1-deoxy-D-xylulose 5-phosphate is of most crucial significance for a number of commercial purposes:

- (1) It may be used as a key intermediate for commercial screening procedures regarding potential inhibitors of downstream enzymes in the biosynthesis of the alternative terpenoid pathway.
- (2) It may be used as a key intermediate for the *in vitro* production of terpenoids or of intermediates thereof.
- (3) It occurs *in vivo* in the biosynthesis of terpenoids as an enzymatic condensation product of glyceraldehyde 3-phosphate and pyruvate. The latter are central intermediates of the metabolism and obligatory starting materials for numerous biosynthetic pathways. Therefore, it is desirable to generate a high level of 1-deoxy-D-xylulose 5-phosphate *in vivo* from an exogenous source and thus independent from the pools of glyceraldehyde 3-phosphate and pyruvate for boosting the biosynthesis of terpenoids or of intermediates thereof in microorganisms or cell cultures that are either naturally or recombinantly endowed with the pathway of interest without influencing the basic intermediary metabolism of the cells.
- (4) 1-Deoxy-D-xylulose 5-phosphate can be generated from 1-deoxy-D-xylulose by the catalytic action of the *xylB* gene product. Using recombinant strains comprising the *xylB* gene the reaction occurs *in vivo* and exogenous 1-deoxy-D-xylulose is converted into intracellular 1-deoxy-D-xylulose 5-phosphate at high rates.
- (5) 1-DXP can be generated from glucose by the catalytic action of glycolytic enzymes and DXP-synthase. Using recombinant strains comprising the *dxs* gene, the reaction occurs *in vivo* and exogenous glucose is converted to intracellular 1-DXP at high rates.

It is an aspect of the invention to use 1-deoxy-D-xylulose as a precursor in order to boost the rates of biosynthesis of 1-deoxy-D-xylulose 5-phosphate-dependent pathways. 1-Deoxy-D-xylulose can be prepared by various published procedures (Blagg and Poulter, 1999; Kennedy et al., 1995; Piel and Boland, 1997; Shono et al., 1983; Giner, 1998).

It is an aspect of the present invention to use 1-deoxy-D-xylulose in various isotopically labelled forms. It may be labelled by radioactive isotopes or non-radioactive isotopes of C (¹³C or ¹⁴C), H (D or T) or O (¹⁷O or ¹⁸O) in any combination.

Isotope-labelled 1-deoxy-D-xylulose may be prepared enzymatically using 1-deoxy-D-xylulose 5-phosphate synthase of *Bacillus subtilis* and commercially available glycolytic enzymes and phosphatase from isotope-labelled glucose and/or pyruvate (PCT/EP00/07548).

1-Deoxy-D-xylulose may be used as a free acid or as a salt, preferably as an alkaline (e. g., lithium, sodium, potassium) salt or as an ammonium or amine salt.

It is an aspect of the present invention to use recombinant cells, cell cultures, or organisms or parts thereof for the formation of biosynthetic products or intermediates or enzymes or for the screening for antimicrobials, antimalarials or herbicides.

For carrying out the present invention various techniques in molecular biology, microbiology and recombinant DNA technology are used which are comprehensively described in Sambrook et al., Molecular Cloning, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; in DNA Cloning: A Practical Approach, Vol. 1 and 2, 1985 (D. N. Glover, ed.); in Oligonucleotide Synthesis, 1984 (M. L. Gait, ed.); and in Transcription and Translation (Hames and Higgins, eds.).

Nucleic acids

The present invention comprises nucleic acids which include prokaryotic, protozoal and plant sequences and derived sequences. A derived sequence relates to a nucleic acid sequence corresponding to a region of the sequence or orthologs thereof or complementary to "sequence-conservative" or "function-conservative" variants thereof.

Sequences may be isolated by well known techniques or are commercially available (Clontech, Palo Alto, CA; Stratagene, LaJolla, CA). Alternatively, PCR-based methods can be used for

amplifying related sequence from cDNA or genomic DNA.

The nucleic acids of the present invention comprise purine and pyrimidine containing polymers in various amounts, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribopolydeoxyribonucleotides. The nucleic acids may be isolated directly from cells. Alternatively, PCR may be used for the preparation of the nucleic acids by use of chemical synthesized strands or by genomic material as template. The primers used in PCR may be synthesized by using the sequence information provided by the present invention or from the database and additionally may be constructed with optionally new restriction sites in order to ease the cloning in a vector for recombinant expression.

The nucleic acids of the present invention may be flanked by natural regulation sequences or may be associated with heterologous sequences, including promoter, enhancer, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3' noncoding regions or similar. The nucleic acids may be modified on basis of well known methods. Non-limiting examples for these modifications are methylations, "Caps", substitution of one or more natural nucleotides with an analogue, and internucleotide modification, i.e. those with uncharged bond (i.e. methylphosphonates, phosphotriester, phosphoramidates, carbamates, etc.) and with charged bond (i.e. phosphorothiactes, etc.). Nucleic acids may carry additional kovalent bound units such as proteins (i.e. nucleases, toxins, antibodies, signalpeptides, poly-L-lysine, etc.), intercalators (i.e. acridine, psoralene, etc.), chelators (i.e. metals, radioactive metals, iron, oxidative metals, etc.) and alkylators. The nucleic acids may be derived by formation of a methyl- or ethylphosphotriester bond or of a alkylphosphoramidate bond. Further, the nucleic acids of the present invention may be modified by labeling, which give an either directly or indirectly detectable signal. Examples for these labeling include radioisotopes, fluorescent molecules, biotin and so on.

Vectors

The invention provides nucleic acid vectors, which comprise the sequences provided by the present invention or derivatives thereof. Various vectors, including plasmids or vectors for fungi have been described for the replication and/or expression in various eucaryotic and prokaryotic hosts. High copy replication vectors are preferred for the purposes of the invention. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids (Invitrogen, San Diego, CA), pET plasmids (Novagen, Inc., Madison, WI) or pRSET or pREP (Invitrogen) and various suitable host cells on basis of well known techniques. Recombinant cloning vectors comprise

often more than one replication system for the cloning and expression, one or more marker for the selection in the host; i.e. antibiotic resistance and one or more expression cartridge. Suitable hosts may be transformed/transfected/infected by a method as suitable including electroporation, CaCl_2 -mediated DNA incorporation, tungae infection, microinjection, microbombardment or other established methods.

Suitable hosts include bacteria, archaebacteriae, fungi, notable yeast, plants, notably *Arabidopsis thaliana*, *Mentha piperita* or *Taxus* sp. and animal cells, notably mammalian cells. Most important are *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Schizosaccharomyces pombe*, SF9 cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells and immortalized myeloid and lymphoid mammalian cells. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus and so on. A great number of transcription, initiation (including ribosomal binding sites) and termination regulation regions have been isolated and there efficiency for the transcription and translation of heterologous proteins has been demonstrated in various hosts. Examples for these regions, methods for the isolation, the way for using are well known. Under suitable conditions for expression host cells may be used as source for the recombinant synthesized proteins.

Expression systems

Preferable vectors may include a transcription element (that is a promoter), functionally connected with the enzyme domain. Optionally, the promoter may include parts of operator region and/or ribosomal binding sites. Non-limiting examples for bacterial promoters, which are compatible with *E. coli*, include: *trc* promoter, β -lactamase (penicillinase) promoter; lactose promoter, tryptophan (*trp*) promoter, arabinose BAD operon-promoter, lambda-derived P1 promoter and N gene ribosomal binding site and the hybrid Tac promoter, derived from sequences of *trp* and *lac* UV5 promoters. Non-limiting examples for yeast promoters include 3-phosphoglycerate kinase promoter, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GAL1) promoter, galactoepimerase promoter and alcoholdehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limiting viral promoters such as i.e. simian virus 40 (SV40), rous sarcoma virus (RSV), adenovirus (ADV) and bovine papilloma virus (BPV). Mammalian cells may also need terminator sequences and poly-A sequences and enhancer sequences, which may increase the expression. Sequences, which amplify the genes, may also be preferred. Further on,

sequences may be included, which ease the secretion of the recombinant protein from the cell, which may be but non-limiting a bacterial, yeast or animal cell, such as i.e. a secretion signal sequence and/or prehormon sequence.

It is an important aspect of the invention that the combined recombinant endowment with *xyB* and other gene(s) of the alternative C5-isoprenoid pathway and optionally gene(s) for higher isoprenoids or terpenoids boost(s) these pathways. Preferably, *xyB* is combined with complete sets of genes to convert 1-deoxy-D-xylulose 5-phosphate into the desired intermediate or end products. For intermediates in the C5-isoprenoid pathway, cells are preferably endowed with one of the combinations of genes given in claim 76.

For the genes cited herein, the common *E. coli* designation were used. Other genes from *E. coli* or from other organisms (orthologous genes) may also be used if they have the same functions (function-conservative genes), notably if their gene products catalyze the same reaction. Further, deletion or insertion variants or fusions of these genes with other genes or nucleic acids may be used, as long as these variants are function-conservative. The above genes may be derived from bacteria, protozoa, or from higher or lower plants,

It is another important aspect of the invention that the function of *gcpE* as following immediately downstream from *ispF* has been determined. Our findings show that the *gcpE* gene product is involved in the formation of the novel compound 1-hydroxy-2-methyl-2-butenyl 4-diphosphate from 2C-methyl-D-erythritol 2,4-cyclodiphosphate. Therefore, we rename *gcpE* in *ispG*.

In a further aspect of the invention it was shown that the gene product of *gcpE* is involved in the formation of the *E*-isomer of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate from 2C-methyl-D-erythritol 2,4-cyclodiphosphate by comparison with chemically synthesized (*E*)- and (*Z*)-isomers of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate. Therefore, this invention further pertains to the (*E*) and (*Z*) isomers of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate salts or protonated forms thereof.

It is another important aspect of the invention that the function of *lytB* has been determined as following immediately downstream from *ispG*. Therefore, it is renamed *ispH*. It is our finding

that *ispH* is involved in the conversion of (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate into isopentenyl 4-diphosphate and/or dimethylallyl 4-diphosphate.

It should be understood that „1-hydroxy-2-methyl-2-but enyl 4-phosphate“ and „1-hydroxy-2-methyl-2-but enyl 4-diphosphate“ comprise the free phosphoric and diphosphoric acids, respectively, and the singly or multiply deprotonated forms thereof, i.e. salts which may be salts of any cation (including Na, K, NH₄⁺, Li, Mg, Ca, Zn, Mn, and Co cations). The protonation state of (di)phosphates and phosphate derivatives or their conjugated acids in aqueous solution depends on the pH value of the solution, as is known to persons skilled in the art. The same applies to other phosphates or phosphate derivatives.

In another aspect of the invention, (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate has been successfully incorporated into the lipid soluble fraction of *Capsicum annuum* chromoplasts. A ¹⁴C label of this compound was incorporated into the geranylgeraniol, β -carotene, phytoene and phytofluene fractions of *C. annuum* chromoplasts establishing (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate as intermediate of the non-mevalonate pathway downstream from 2C-methyl-D-erythritol 2,4-cyclodiphosphate and upstream from isopentenyl diphosphate.

It is another aspect of the invention that *xylB* can be combined with *gcpE* and optionally other genes of the alternative C5 isoprenoid pathway and/or of the higher isoprenoid pathways in vector(s) for recombinant engineering.

As a consequence of our findings regarding *gcpE* (now *ispG*) it follows that the gene *lytB* operates downstream of *gcpE* and thus in service of the conversion of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate to IPP and/or DMAPP. Therefore, it is another aspect of the invention to combine the gene *lytB* with *xylB* and optionally other genes of the common C5-isoprenoid pathway or of a higher isoprenoid pathway.

Our finding allows the efficient formation or production of intermediates or products of the isoprenoid pathway with any desired labelling, notably the following intermediates: 2C-methyl-D-erythritol 4-phosphate; 4-diphosphocytidyl-2C-methyl-D-erythritol; 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; 2C-methyl-D-erythritol 2,4-

cyclodiphosphate; 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, isopentenyl diphosphate; dimethylallyl diphosphate.

The formation of end products of the terpenoid pathway (e. g., β -carotene, zeaxanthine, paclitaxel, menthol, menthone, cannabinoids), may be boosted following the process of the invention.

The strains harbouring the recombinant plasmids can be cultivated in conventional culture media, preferably in terrific broth medium, at 15 to 40 °C. The preferred temperature is 37 °C. The *E. coli* strains are induced with 0.5 to 2 mM isopropyl- β -D-thiogalactoside (IPTG) at an optical density at 600 nm from 0.5 to 5. The cells are incubated after addition of 1-deoxy-D-xylulose at a concentration of 0.001 mM to 1 M preferably at a concentration of 0.01 to 30 mM for 30 min to 15 h, preferably 1 to 5 h.

It has been found that the process of producing isoprenoid intermediates or products by the genetically engineered organisms of the invention can be boosted by supplying a source for CTP, for example cytidine and/or uridine and/or cytosine and/or uracil and/or ribose and/or ribose 5-phosphate and/or any biosynthetic precursors of CTP at a concentration of 0.01 to 10 mM, preferably at a concentration of 0.3 to 1 mM, and/or by supplying a source for phosphorylation activity, for example glycerol 3-phosphate and/or phosphoenolpyruvate and/or ribose 5-phosphate at a concentration of 0.1 to 100 mM, preferably at a concentration of 0.5 to 10 mM and/or inorganic phosphate and/or inorganic pyrophosphate at a concentration of 1 to 500 mM, preferably at a concentration of 10 to 100 mM and/or any organic phosphate and/or pyrophosphate, and/or by supplying a source for reduction equivalents, for example 0.1 to 1000 mM, preferably 10 to 1000 mM, lactate and/or succinate and/or glycerol and/or glucose and/or lipids at a concentration of 0.1 to 100 mM, preferably at a concentration of 0.5 to 10 mM. A particularly efficient production process is specified in claims 72 and 80 to 84.

This process can also be used with great advantages for screening for inhibitors of the enzymes involved or of downstream enzymes, dependent on the choice of the isoprenoid intermediate or product for detection. The enzymes *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG* (formerly *gcpE*) and *ispH* (formerly *lytB*) do not occur in animals. Therefore inhibitors against *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG* (formerly *gcpE*) and *ispH* (formerly *lytB*) have great value as (a) herbicides against weed plants or algae; (b) antibiotic agents against pathogenic bacteria; (c) agents against protozoa, like *Plasmodium falciparum*, the causative pathogen of malaria.

The activity of the said enzymes can be detected (in the presence or absence of a potential inhibitor) by measuring either the formation of a product or the consumption of an intermediate, preferably by TLC, HPLC or NMR.

With the finding that 1-hydroxy-2-methyl-2-but enyl 4-diphosphate is an intermediate of the non-mevalonate terpenoid pathway we have acquired essential determinants of the structure of inhibitors. Namely, the structures of a subset of inhibitors should be similar to at least a portion of the starting compound or the product or the transition state between the starting compound e.g. 2C-methyl-D-erythritol 2,4-cyclodiphosphate and the product e.g. 1-hydroxy-2-methyl-2-but enyl 4-diphosphate.

This invention discloses novel compounds, or salts thereof, of the following formula I:



whereby R¹ and R² are different from each other and one of R¹ and R² is hydrogen and the other is selected from the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂, -CH₂-O-PO(OH)₂, and -CH₂OH, and whereby A stands for -CH₂OH or -CHO. These compounds may be isotope-labelled.

In formula I, A preferably stands for -CH₂OH.

Among R¹ and R², R¹ is preferably hydrogen and R² is preferably selected from the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂ and -CH₂-O-PO(OH)₂.

In the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂ and -CH₂-O-PO(OH)₂, -CH₂-O-PO(OH)-O-PO(OH)₂ is preferred.

If a compound of formula I is a salt, it may e.g. be a lithium, sodium, potassium, magnesium, ammonium, manganese salt. These salts may derive from a single or from multiple deprotonations from the (di)phosphoric acid moiety.

The novel compounds disclosed herein are useful for various applications e.g. for screening for genes, enzymes or inhibitors of the biosynthesis of isoprenoids or terpenoids, either *in vitro* in the presence of an electron donor or *in vivo*.

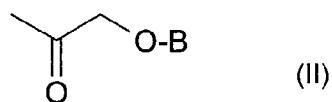
This invention further provides a process for the chemical preparation of a compound of

formula I or a salt thereof:

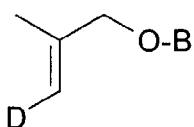


wherein A represents $-\text{CH}_2\text{OH}$ and R¹ and R² are different from each other and one of R¹ and R² is hydrogen and the other is $-\text{CH}_2\text{O-PO(OH)-O-PO(OH)}_2$, $-\text{CH}_2\text{O-PO(OH)}_2$ or $-\text{CH}_2\text{OH}$ by the following steps:

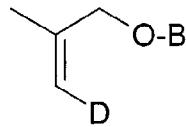
(a) converting a compound of the following formula (II):



wherein B is a protective group into a compound of the following formula (III) or (IV):



(III)



(IV)

by a Wittig or Horner reagent, wherein the group D is a precursor group convertible reductively to a $-\text{CH}_2\text{OH}$ group;

- (b) reductively converting group D to a $-\text{CH}_2\text{OH}$ group;
- (c) optionally converting group $-\text{CH}_2\text{OH}$ obtained in step (b) into $-\text{CH}_2\text{O-PO(OH)-O-PO(OH)}_2$ or $-\text{CH}_2\text{O-PO(OH)}_2$ or salts thereof in a manner known per se;
- (d) optionally conversion to a desired salt;
- (e) removing the protective group B.

In the above process, said protective group B may be any group that allows to regenerate an hydroxy group at the position it is attached to. Said protective group B is preferably stable under the conditions of step (a) to step (d). Said protective group B is removed in step (e) of said process in order to generate a hydroxy group. Protective groups for hydroxy groups are known to the skilled person. Group B may for example form an acetal group together with the

remaining moiety of the compound of formula (II), (III) or (IV). Acetals can be hydrolysed under acidic conditions. Most preferably, group B is a 2-tetrahydropyranyl group.

In the above process, said group D is a precursor group convertible reductively to a -CH₂-OH group. Group D may be a derivative of a carbon acid. Examples of such a group include alkoxy carbonyl and aminocarbonyl groups. Said aminocarbonyl groups may be substituted at the amino group with one or two alkyl groups. It is most preferred to use alkoxy carbonyl groups. The alkyl group of said alkoxy carbonyl groups or said alkyl groups of said aminocarbonyl groups may be a linear or branched alkyl groups which may be singly or multiply substituted. Preferred are C₁-C₆ alkyl groups like methyl, ethyl, propyl, butyl, pentyl or hexyl groups. Most preferred are methyl or ethyl groups. The most preferred example of said group D is an ethoxycarbonyl group.

Said compound of formula (II) may be prepared by protecting the hydroxy group of hydroxy acetone with said group B. If group B is a tetrahydropyranyl group, the compound of formula (II) may be prepared from hydroxy acetone and 3,4-dihydro-2H-pyran, preferably employing pyridinium toluene-4-sulfonate as a catalyst. A specific method for preparing acetonyl tetrahydropyranyl ether is described in example 24.

In step (a) of said process, the compound of formula (II) is converted to a compound of formula (III) or (IV) by a Wittig or a Horner reagent. Wittig-type reactions and reagents are known to skilled persons (see e.g. Watanabe *et al.* 1996 and references cited therein). Common Wittig reagents to be used for the above process are methylenetriphenylphosphoranes which may be substituted at the methylene group. For the above process of this invention, a methylenetriphenylphosphorane is employed which is substituted with the above-defined group D at the methylene group. Such Wittig reagents are commercially available or can be prepared according to known methods.

The olefin produced in step (a) may be formed as a mixture of the cis/trans isomers of formulas (III) and (IV). If one of said isomers is preferred, it may be enriched or separated from the other isomer by methods known in the art, preferably by chromatography. Alternatively, a separation of said isomers may be carried out after one of the following steps (b) to (e).

In step (b) of the above process, group D of the compound of formula (III) or (IV) or a mixture of said compounds is reductively converted to a -CH₂-OH group. Various methods are known

in the art to perform such a reduction. Conditions are chosen such that group D is reduced whereas the olefin moiety is not. Examples for reductants to be used in this step are molecular hydrogen or metal hydrides. Examples for useful metal hydrides include boron hydrides like sodium borohydride, aluminium hydrides like lithium aluminium hydride or diisobutyl aluminiumhydride (DIBAH), alkali metal or metal earth hydrides like sodium hydride or calcium hydride. Aluminium hydrides are preferred. A specific example for carrying out step (b) is described in example 24.

If the desired end product of said process is a compound of formula (I), wherein R¹ or R² is -CH₂-OH, the compound or mixture of compounds obtained in step (b) may be directly subjected to step (d) or step (e). Preferably, it is subjected to step (e) for removing protective group B. If the desired end product of said process is a compound of formula (I), wherein R¹ or R² is -CH₂-O-PO(OH)-O-PO(OH)₂ or -CH₂-O-PO(OH)₂, compound or mixture of compounds obtained in step (b) is subjected to step (c) of said process for converting -CH₂-OH group obtained in step (b) into a -CH₂-O-PO(OH)-O-PO(OH)₂ or a -CH₂-O-PO(OH)₂ group.

Step (c) may be carried in several ways which are known to the skilled person. Step (c) may comprise substituting the hydroxy group of said -CH₂-OH group obtained in step (b) by a leaving group. Step (c) may comprise converting said -CH₂-OH group to a -CH₂-halide group by a halogenating agent. A sulfuric, sulfonic or phosphoric acid halogenide may be employed as halogenating agent. Tosyl chloride is most preferred. Said halide may be fluoride, chloride, bromide or iodide, preferably chloride. The compound carrying said -CH₂-halide group is preferably isolated. Said leaving group may further be created by reacting said -CH₂-OH group obtained in step (b) with a sulfonic acid halide, preferably tosyl chloride.

Said intermediate having said leaving group may then be reacted with phosphoric or diphosphoric acid or singly or multiply deprotonated forms thereof. Preferably an alkylammonium salt of phosphoric or diphosphoric acid is used, more preferably a tetraalkylammonium salt, and most preferably a tetra-butylammonium salt. A polar aprotic solvent is preferred for this reaction. Preferably, the compound or mixture of compounds obtained is purified according to standard procedures. A specific example for carrying out step (c) is described in example 24.

In step (d), the compound or mixture of compounds obtained in step (c) may be converted to a desired salt. Methods for carrying out step (d) are well known. Such methods may comprise

adjusting the pH of an aqueous solution with an appropriate acid or salt to a desired pH value.

In step (e), the protective group B of a compound obtained in one of steps (b) to (d) is removed in order to obtain a compound of formula (I) wherein A is -CH₂-OH. The method for removing a protective group depends on the type of the protective group. Such methods are well known. If the protective groups forms an acetal, removing said protecting group may be achieved by acid hydrolysis (see example 24).

This invention provides protein in a form that is functional for the enzymatic conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-but enyl 4-diphosphate notably in its (*E*)-form, preferably in the presence of NADH and/or NADPH and/or in the presence of Co²⁺. Said enzyme preferably has a sequence encoded by the *ispG* (formerly *gcpE*) gene of *E. coli* or a function-conservative homologue of said sequence, i.e. said homologue is capable of performing the same function as said protein. For many applications of said protein, it may be expressed and purified as a fusion protein, notably a fusion with maltose binding protein. In this way, enzymatically active protein may be readily obtained.

This invention further provides a protein in a form that is functional for the enzymatic conversion of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably in its (*E*)-form, to isopentenyl diphosphate and/or dimethylallyl diphosphate. Said protein preferably requires FAD and NAD(P)H for said functionality. Further, said protein may require a metal ion selected from the group of manganese, iron, cobalt, or nickel ion. Said protein preferably has a sequence encoded by the *ispH* (formerly *lytB*) gene of *E. coli* or a function-conservative homologue of said sequence. For many applications of said protein, it may be expressed and purified as a fusion protein, notably a fusion with maltose binding protein. In this way, enzymatically active protein may be readily obtained.

The above proteins may be plant proteins, notably from *Arabidopsis thaliana*, bacterial proteins, notably from *E. coli*, or protozoal proteins, notably from *Plasmodium falciparum*.

The invention further provides a purified isolated nucleic acid encoding one or both of the above proteins with or without introns. Further, the invention provides a DNA expression vector containing the sequence of said purified isolated nucleic acid.

The invention further provides cells, cell cultures, organisms or parts thereof recombinantly endowed with the sequence of said purified isolated nucleic acid or with said DNA expression vector, wherein said cell is selected from the group consisting of bacterial, protozoal, fungal, plant, insect and mammalian cells. Said cells, cell cultures, organisms or parts thereof may further be endowed with at least one gene selected from the following group: *dxs*, *dxr*, *ispD* (formerly *ygbP*); *ispE* (formerly *ychB*); *ispF* (formerly *ygbB*) of *E. coli* or a function-conservative homologue thereof, or a function-conservative fusion, deletion or insertion variant of any of the above genes.

The invention further provides cells, cell cultures, or organisms or parts thereof transformed or transfected for an increased rate of formation of 1-hydroxy-2-methyl-2-butanyl 4-diphosphate, notably in its (*E*)-form, compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection. The transformation or transfection preferably comprises endowment with the *gcpE* gene of *E. coli* or with a function-conservative homologue from an other organism, e.g. plant or protozoal organism.

The invention also provides cells, cell cultures, or organisms or parts thereof transformed or transfected for an increased rate of conversion of (*E*)-1-hydroxy-2-methyl-2-butanyl 4-diphosphate to isopentenyl diphosphate and/or dimethylallyl diphosphate compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection. The transformation or transfection preferably comprises endowment with the *lytB* gene of *E. coli* or with a function-conservative homologue from an other organism, e.g. plant or protozoal organism.

The invention provides also cells, cell cultures, or organisms or parts thereof transformed or transfected for an increased expression level of the protein of one of claims 1 to 4 and/or the protein of one of claims 5 to 8 compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection.

Moreover, the invention provides a method of altering the expression level of the gene product(s) of *ispG* and/or *ispH* or function-conservative homologues from other organisms or variants thereof in cells comprising

- (a) transforming host cells with the *ispG* and/or *ispH* gene,
- (b) growing the transformed host cells of step (a) under conditions that are suitable for the

efficient expression of *ispG* and/or *ispH*, resulting in production of altered levels of the *ispG* and/or *ispH* gene product(s) in the transformed cells relative to expression levels of untransformed cells.

Furthermore, the invention provides a method of identifying an inhibitor of an enzyme functional for the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, notably its *E*-form, of the non-mevalonate isoprenoid pathway by the following steps:

- (a) incubating a mixture containing said enzyme with its, optionally isotope-labeled, substrate 2C-methyl-D-erythritol-2,4-cyclodiphosphate under conditions suitable for said conversion in the presence and in the absence of a potential inhibitor,
- (b) subsequently determining the concentration of 2C-methyl-D-erythritol 2,4-cyclodiphosphate and/or 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, and
- (c) comparing the concentration in the presence and in the absence of said potential inhibitor.

Furthermore, the invention provides a method of identifying an inhibitor of an enzyme functional for the conversion of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, notably its *E*-form, to isopentenyl diphosphate or dimethylallyl diphosphate of the non-mevalonate isoprenoid pathway by the following steps:

- (a) incubating a mixture containing said enzyme with its, optionally isotope-labeled, substrate 1-hydroxy-2-methyl-2-butenyl 4-diphosphate under conditions suitable for said conversion in the presence and in the absence of a potential inhibitor, whereby said mixture preferably contains FAD,
- (b) determining the concentration of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate and/or isopentenyl diphosphate or dimethylallyl diphosphate, and
- (c) comparing the concentration in the presence and in the absence of said potential inhibitor.

The above methods of identifying an inhibitor are preferably carried out by following the consumption of NADPH or NADH making use of its characteristic absorbance spectrum. Alternatively, the fluorescence of NADH or NADPH can be followed when excited around 340 nm. The above methods of identifying an inhibitor may advantageously be performed as high-

throughput screening assays for inhibitors, notably in combination with photometric detection of the consumption of NADH or NADPH. Further, one or more flavin analogues (e.g. FAD, FMN) may be added to the incubation mixtures in said methods, preferably in catalytic amounts. Most preferred is the addition of FAD. Said enzymes may be employed in said methods as fusion proteins with maltose binding protein (examples 38 to 41, 44, 45), which allows straightforward expression and purification of said enzymes in enzymatically active form. Further embodiments of said methods of identifying are defined in the subclaims to these methods.

It is known that intermediates of the non-mevalonate pathway are responsible for $\gamma\delta$ T cell activation by various pathogenic bacteria. $\gamma\delta$ T cell activation is followed by T cell proliferation, secretion of cytokines and chemokines and is very likely crucial for regulating the immune response following pathogen infection (Altincicek *et al.*, 2001 and references cited therein). Recently, it was shown that *E. coli* strains lost the ability to stimulate $\gamma\delta$ T cells when the *dxr* or the *gcpE* gene was knocked out, strongly indicating that an intermediate downstream of *gcpE* and upstream of isopentenyl pyrophosphate exhibits the most potent antigenic activity (Altincicek *et al.*, 2001). However, the intermediate produced by the *gcpE* gene product in the pathway has been unknown. Herein, this intermediate has surprisingly been identified as an hitherto unprecedented compound, which opens up a whole range of novel applications for this compound.

The compounds of formula I can be used as immunomodulatory or immunostimulating agents, e.g. for activating $\gamma\delta$ T cells. Immunomodulation via $\gamma\delta$ T cell activation by said compounds may prove useful not only to support combat against pathogens but for various conditions for which a stimulation of the immune system is desirable. The novel compounds of the invention may therefore be used for medical treatment of pathogen infections. Such a treatment stimulates the activity of the immune system against the pathogen. Preferably, the compound wherein $R^1=H$ and/or A is $-CH_2OH$ is used for this application. Alternatively, the oxidation product with A = CHO may prove to be highly active. Among the compounds of formula I, the one with the highest or most suitable $\gamma\delta$ T cell stimulating activity may be selected in a test system known in the art (e.g. that described by Altincicek *et al.*, 2001). Importantly, since the compounds of the invention do not act as antibiotics, development of resistancies is not a problem for the method of treatment disclosed herein.

In an advantageous embodiment, said compounds may be combined with an antibiotically active compound for treating a pathogen infection. Such a treatment combines the advantages of inhibiting pathogen proliferation by an antibiotic and stimulating the immune system against the pathogen resulting in a much faster and more efficient treatment. Such an antibiotically active compound may be a bacteriostatic antibiotic (e.g. tetracyclines).

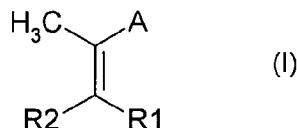
Therefore, the novel compounds of this invention may be used for the preparation of a medicament. The invention further pertains to a pharmaceutical composition containing a compound of formula I and a pharmaceutically acceptable carrier. Said pharmaceutical composition may further contain an antibiotically active compound as mentioned above.

This invention further comprises antibodies against the compounds of formula I. Said antibodies may be polyclonal or monoclonal and may be raised according to conventional techniques. Raising such antibodies will comprise coupling of a compound of formula I has hapten to a macromolecular carrier like a protein in order to be immunogenic. Such an immunogenic compound of formula I may further be used as a vaccine.

The antibodies of the invention may be used for detecting a compound of formula I. Since said compounds are produced by organisms having the non-mevalonate isoprenoid pathway, such organisms may be detected using said antibodies. Preferably, such organisms may be detected in body fluids in a diagnostic method, thereby indicating an infection by a pathogen having the non-mevalonate pathway. A positive result in such a diagnostic method may at the same time indicate possible treatment by the compounds of the invention.

When an antibody of the invention is used for detecting a compound of formula I, it is preferably labelled to allow photometric detection and/or immobilized to a support. Such methods are well-known in the art.

This invention further provides a process for the chemical preparation of a compound of formula I or a salt thereof (see Fig. 7):



wherein A represents -CH₂OH or -CHO, R¹ is hydrogen, and R² is -CH₂-O-PO(OH)-O-

PO(OH)₂, -CH₂-O-PO(OH)₂ or -CH₂-OH by the following steps:

- (a) converting 2-methyl-2-vinyl-oxiran into 4-chloro-2-methyl-2-buten-1-al;
- (b) converting 4-chloro-2-methyl-2-buten-1-al to its acetal;
- (c) substituting the chlorine atom in the product of step (b) by a hydroxyl group, a phosphate group or a pyrophosphate group;
- (d) hydrolysing the acetal obtained in step (c) to produce an aldehyde group;
- (e) optionally converting the aldehyde group of the product of step (d) to a -CH₂OH group.

Preferred embodiments of this process are defined in the subclaims and are exemplified in example 42.

The invention will now be described in detail with reference to specific examples.

Example 1

Construction of a vector carrying the *xyI/B* gene of *Escherichia coli* capable for transcription and expression of D-xylulokinase

Chromosomal DNA from *Escherichia coli* strain XL1-Blue (Bullock et al. 1987; commercial source: Stratagene, LaJolla, CA, USA) is isolated according to a method described by Meade et al. 1982.

The *E. coli* ORF *xyI/B* (accession no. gb AE000433) from base pair (bp) position 8596 to 10144 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CCGTCGGAATTCGAGGAGAAATTAACCATGTATATCGGGATAGATCTTGG-3', 10 pmol of the primer 5'-GCAGTGAAGCTTTACGCCATTAATGGCAGAAGTTGC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec, Seraing, Belgium) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 75 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden, Germany).

1.0 μ g of the vector pBluescript SK^{II}⁻ (Stratagene) and 0.5 μ g of the purified PCR product are digested with *Eco*RI and *Hind*III in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (New England Biolabs, Frankfurt am Main, Germany (NEB)) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 20 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pBSxylB. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells according to a method described by Dower et al., 1988. The plasmid pBSxylB is isolated with the plasmid

isolation kit from Qiagen.

The DNA insert of the plasmid pBSxylB is sequenced by the automated dideoxynucleotide method (Sanger *et al.*, 1992) using an ABI Prism 377™ DNA sequencer from Perkin Elmer (Norwalk, USA) with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions (Foster city, USA). It is identical with the DNA sequence of the database entry (gb AE000433).

Example 2

Construction of a vector carrying the *xylB* and *dxr* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase and DXP reductoisomerase

The *E. coli* ORF *dxr* (accession no. gb AE000126) from base pair (bp) position 9887 to 11083 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CTAGCCAAGCTTGAGGAGAAATTACCATGAAGCAACTCACCATTCTGG-3', 10 pmol of the primer 5'-GGAGATGTCGACTCAGCTTGCAGACGC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 75 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.2 µg of the vector pBSxylB (Example 1) and 0.6 µg of the purified PCR product are digested with *Hind*III and *Sal*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 18 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pBSxylBdxr. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBSxylBdxr is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBSxylBdxr is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000126).

The DNA sequence of the vector construct pBSxylBdxr is shown in Annex A.

Example 3

Construction of a vector carrying the *xyI**B*, *dxr* and *ispD* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase and CDP-ME synthase

The *E. coli* ORF *ispD* (accession no. gb AE000358) from base pair (bp) position 6754 to 7464 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CCGGGAGTCGACGAGGAGAAATTAAACCATGGCAACCACCTCATTTGGATG-3', 10 pmol of the primer 5'-GTCCAACTCGAGTTATGTATTCTCCTTGATGG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 30 sec at 94 °C, 30 sec at 50 °C and 45 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.5 μ g of the vector pBSxylBdxr (Example 2) and 0.8 μ g of the purified PCR product are digested with *Sall* and *Xhol* in order to produce DNA fragments with overlapping ends. The

restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 12 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pBSxylBdxrispD. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBSxylBdxrispD is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBSxylBdxrispD is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000126).

The DNA sequence of the vector construct pBSxylBdxrispD is shown in Annex B.

Example 4

Construction of a vector carrying the *xytB*, *dxr*, *ispD* and *ispF* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase, CDP-ME synthase, and cMEPP synthase

The *E. coli* ORF's *ispD* and *ispF* (accession no. gb AE000358) from base pair (bp) position 6275 to 7464 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CCGGGAGTCGACGAGGAGAAATTAAACCATGGCAACCACTCATTGGATG-3', 10 pmol of the primer 5'-TATCAACTCGAGTCATTTGTTGCCTTAATGAG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 75 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.4 µg of the vector pBSxylBdxr (Example 2) and 0.7 µg of the purified PCR product are digested with *Sall* and *Xhol* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 18 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pBSxylBdxriscDF. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBSxylBdxriscDF is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBSxylBdxriscDF is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000126).

Example 5

Construction of a vector carrying the *xylB*, *dxr*, *ispD*, *ispE* and *ispF* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase, CDP-ME synthase, CDP-ME kinase and cMEPP synthase

The *E. coli* ORF *ispE* (accession no. gb AE000219) from base pair (bp) position 5720 to 6571 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GCGAACCTCGAGGAGGGAAATTAAACCATGCAGACACAGTGGCCC-3', 10 pmol of the primer 5'-CCTGACGGTACCTAAAGCATGGCTCTGTGC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.2 µg of the vector pBSxylBdxrispDF (Example 4) and 0.6 µg of the purified PCR product are digested with *Xhol* and *KpnI* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 15 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pBScyclo. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScyclo is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScyclo is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000219). The DNA sequence of the vector construct pBScyclo is shown in Annex C.

Example 6

Construction of a vector carrying the *gcpE* gene of *Escherichia coli* capable for its transcription and expression

The *E. coli* ORF *gcpE* (accession no. gb AE000338) from base pair (bp) position 372 to 1204 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CGTACCGGATCCGAGGAGAAATTAAACCATGCATAACCAGGGCTCCAATT-3', 10 pmol of the primer 5'-CCCATCGTCGACTTATTTTCAACCTGCTAACGTC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of

100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.0 μ g of the vector pACYC184 (Chang and Cohen 1978, NEB) and 0.7 μ g of the purified PCR product are digested with *Bam*H1 and *Sal*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 20 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pACYCgcpE. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYCgcpE is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYCgcpE is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000338).

The DNA sequence of the vector construct pACYCgcpE is shown in Annex D.

Example 7

Construction of vectors carrying a carotenoid operon from *Erwinia uredovora* capable for the *in vivo* production of β -carotene

The open reading frames *crtY*, *crtI* and *crtB* of a carotenoid operon from *Erwinia uredovora* (accession no. gb D90087) from base pair (bp) position 2372 to 6005 is amplified by PCR

using chromosomal *E. uredovora* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CATTGAGAAGCTTATGTGCACCG-3', 10 pmol of the primer 5'-CTCCGGGGTCGACATGGCGC-3', 40 ng of chromosomal DNA of *E. uredovora*, 8 U of Taq DNA polymerase (Eurogentec), 20 nmol of dNTPs, Taq Extender (Stratagene) in a total volume of 100 μ l 1x Taq Extender buffer (Stratagene).

The mixture is denatured for 3 min at 94 °C. Then 40 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 300 sec at 72 °C followed. After further incubation for 20 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden, Germany).

1.0 μ g of the vector pBluescript SKII⁺ (Stratagene) and 2.0 μ g of the purified PCR product are digested with *Hind*III and *Sall* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 40 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pBScaro34. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScaro34 is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScaro34 is sequenced by the automated dideoxynucleotide method using an ABI Prism 377TM DNA sequencer from Perkin Elmer with the ABI PrismTM Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb D90087).

The *E. uredovora* ORF *crtE* (accession no. gb D90087) from base pair (bp) position 175 to 1148 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-CCGCATCTTCCAATTGCCG-3', 10 pmol of the primer 5'-ATGCAGCAAGCTTAAGCTGACGGC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA

polymerase (Eurogentec, Seraing, Belgium) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden, Germany).

1.5 μ g of the vector pBScaro34 (see above) is digested with EcoRI and HindIII and 0.6 μ g of the purified PCR product are digested with MfeI and HindIII in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 16 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pBScaro14. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScaro14 is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScaro14 is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb D90087). The DNA sequence of the plasmid pBScaro14 is shown in Annex E.

5 μ g of the vector pBScaro14 (see above) is digested with BamHI and SalI. The restriction mixture is prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. The restriction mixture is separated on a agarose gel and the fragments of 2237 and 2341 bp size are purified with the gel extraction kit from Qiagen.

3 μ g of the vector pACYC184 (see above) is digested with BamHI and SalI. The restriction

mixture is prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. The restriction mixture is separated on a agarose gel and the fragment of 3968 bp size is purified with the gel extraction kit from Qiagen.

30 ng of the purified vector DNA and each 25 ng of the purified 2237 and 2341 bp fragments are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYCcaro14. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYCcaro14 is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYCcaro14 is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb D90087). The DNA sequence of the plasmid pACYCcaro14 is shown in Annex F.

Example 8

Enzymatic preparation of [U-¹³C₅]1-deoxy-D-xylulose 5-phosphate

A reaction mixture containing 960 mg of [U-¹³C₆]glucose (5.1 mmol), 6.1 g of ATP (10.2 mmol), 337 mg of thiamine pyrophosphate, 1.14 g of [2,3-¹³C₂]pyruvate (10.2 mmol), 10 mM MgCl₂, 5 mM dithiothreitol in 150 mM Tris hydrochloride, pH 8.0 is prepared. 410 Units of triose phosphate isomerase (from rabbit muscle, Type III-S, E. C. 5.3.1.1., Sigma), 100 U hexokinase (from Bakers Yeast, Type VI, E. C. 2.7.1.1, Sigma), 100 U phosphoglucose isomerase (from Bakers Yeast, Type III, E. C. 5.3.1.9, Sigma), 100 U phosphofructokinase (from *Bacillus stearothermophilus*, Type VII, E. C. 2.7.1.11, Sigma), 50 U aldolase (from rabbit muscle, E. C. 4.1.2.13, Sigma) and 12 U of recombinant DXP synthase from *B. subtilis* are added to a final volume of 315 ml. The reaction mixture is incubated at 37 °C overnight and during incubation the pH is hold at a constant value of 8.0. The reaction is monitored by ¹³C NMR spectroscopy.

Example 9

Enzymatic preparation of [3,4,5-¹³C₃]1-deoxy-D-xylulose 5-phosphate

A solution containing 150 mM Tris hydrochloride, 10 mM MgCl₂, 1.0 g of [U-¹³C₆]glucose (5.4 mmol), 0.23 g (1.5 mmol) of dithiothreitol, 0.3 g (0.7 mmol) of thiamine pyrophosphate, 0.1 g (0.2 mmol) of ATP (disodium salt), and 2.2 g (11mmol) of phosphoenol pyruvate (potassium salt) is adjusted to pH 8.0 by the addition of 8 M sodium hydroxide. 403 U (2.8 mg) of pyruvate kinase (from rabbit muscle, E. C. 2.7.1.40), 410 Units of triose phosphate isomerase (from rabbit muscle, Type III-S, E. C. 5.3.1.1., Sigma), 100 U hexokinase (from Bakers Yeast, Type VI, E. C. 2.7.1.1, Sigma), 100 U phosphoglucose isomerase (from Bakers Yeast, Type III, E. C. 5.3.1.9, Sigma), 100 U phosphofructokinase (from *Bacillus stearothermophilus*, Type VII, E. C. 2.7.1.11, Sigma), 50 U aldolase (from rabbit muscle, E. C. 4.1.2.13, Sigma) and 12 U recombinant DXP synthase from *B. subtilis* are added to a final volume of 300 ml. The reaction mixture is incubated at 37 °C for overnight.

Example 10

Enzymatic preparation of 1-deoxy-D-xylulose

The pH value of the reaction mixture obtained in example 8 or 9 is adjusted to 9.5. Magnesium chloride is added to a concentration of 30 mM. 50 mg (950 Units) of alkaline phosphatase from bovine intestinal mucosa (Sigma, E. C. 3.1.3.1) are added and the reaction mixture is incubated for 16 h. The conversion is monitored by ¹³C-NMR spectroscopy. The pH is adjusted to a value of 7.0 and the solution is centrifuged at 14,000 upm for 5 minutes. Starting from labelled glucose (examples 8 or 9) the overall yield of 1-deoxy-D-xylulose is approximately 50 %.

The supernatant or the lyophilised supernatant is used in incorporation experiments (see examples 11 to 17).

Example 11

Incorporation experiment with recombinant *Escherichia coli* XL1-pBSxylB using [3,4,5-¹³C₃]1-deoxy-D-xylulose

0.2 litre of Luria Bertani (LB) medium containing 36 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harbouring the plasmid pBSxylB (see example 1). The cells are grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.6 the culture is induced with 2 mM IPTG. Two hours after induction with IPTG, 50 ml (0.9 mmol) of

crude [3,4,5-¹³C₃]1-deoxy-D-xylulose (pH 7.0) (see examples 9 and 10), are added. Aliquots of 25 ml are taken at time intervals of 30 minutes and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 µl of 20 mM NaF in D₂O, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. ¹³C NMR spectra of the supernatant are recorded directly, without further purification, with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). The NMR analysis is based on published signal assignments (Wungsintaweekul *et al.*, 2001).

30 min after the addition of [3,4,5-¹³C₃]1-deoxy-D-xylulose, the formation of [3,4,5-¹³C₃]1-deoxy-D-xylulose 5-phosphate can be observed. The maximum yield of [3,4,5-¹³C₃]1-deoxy-D-xylulose 5-phosphate is observed 3-5 h after addition of [3,4,5-¹³C₃]1-deoxy-D-xylulose to the medium. The ¹³C NMR signals reveal a mixture of [3,4,5-¹³C₃]1-deoxy-D-xylulose and [3,4,5-¹³C₃]1-deoxy-D-xylulose-5-phosphate at a molar ratio of approximately 1 : 9. The intracellular concentration of [3,4,5-¹³C₃]1-deoxy-D-xylulose 5-phosphate is estimated as 20 mM by quantitative NMR spectroscopy.

Example 12

Incorporation experiment with recombinant *Escherichia coli* XL1-pBSxylBdxr using [U-¹³C₅]1-deoxy-D-xylulose

0.12 litre of Luria Bertani (LB) medium containing 22 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harbouring plasmid pBSxylBdxr (see example 2). The cells are grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.6 the culture is induced with 2 mM IPTG. Two hours after induction with IPTG, ca. 1.0 mmol of crude [U-¹³C₅]1-deoxy-D-xylulose (pH 7.0) (see examples 8 and 10) are added. Aliquots of 25 ml are taken in time intervals of 1 h and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 µl of 20 mM NaF in D₂O, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the supernatant are recorded directly, without further purification, with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany).

HMQC and HMQC-TOCSY experiments reveal ^1H - ^{13}C and ^1H - ^1H spin systems (Table 1) of [U - $^{13}\text{C}_5$]2C-methyl-D-erythritol 4-phosphate, [U - $^{13}\text{C}_5$]2C-methyl-D-erythritol and [1,2,2',3,4- $^{13}\text{C}_5$]4-diphosphocytidyl-2C-methyl-D-erythritol at a molar ratio of approximately 6.6 : 7 : 1, respectively. The intracellular concentration of [U - $^{13}\text{C}_5$]2C-methyl-D-erythritol 4-phosphate is estimated as 10 mM by quantitative NMR spectroscopy.

The NMR data summarized in Table 1 are identical with published NMR data of the authentic compounds (Takahashi et al., 1998; Rohdich et al., 1999).

Table 1. NMR data of ^{13}C -labeled products in cell extracts of *E. coli* XL1-pBSxylBdxr after feeding of [U - $^{13}\text{C}_5$]1-deoxy-D-xylulose

| Position | Chemical shifts, ppm | | | | | | |
|---|----------------------|------|------|----------|------|------|------|
| | 1 | 1* | 2 | 2-Methyl | 3 | 4 | 4* |
| [U - $^{13}\text{C}_5$]2C-methyl-D-erythritol 4-phosphate | | | | | | | |
| ^{13}C | 66.1 | n.d. | 18.1 | 73.4 | | 648 | |
| ^1H | 3.25 | 3.36 | | 0.93 | 3.56 | 3.62 | 3.81 |
| [U - $^{13}\text{C}_5$]2C-methyl-D-erythritol | | | | | | | |
| ^{13}C | 66.6 | n.d. | 18.0 | 74.6 | | 616 | |
| ^1H | 3.26 | 3.34 | | 0.9 | 3.44 | 3.36 | 3.61 |
| [1,2,2',3,4- $^{13}\text{C}_5$]4-diphosphocytidyl-2C-methyl-D-erythritol | | | | | | | |
| ^{13}C | 66.8 | n.d. | 18.0 | 73.0 | | 667 | |
| ^1H | 3.4 | 3.55 | | 0.9 | 3.6 | 3.74 | 4 |

Example 13

Incorporation experiment with recombinant *Escherichia coli* XL1-pBSxylBdxrispDF using [3,4,5- $^{13}\text{C}_3$]1-deoxy-D-xylulose

0.1 litre of Luria Bertani (LB) medium containing 18 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harbouring the plasmid pBSxylBdxrispDF (see example 4). The cells are grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.5, the culture is induced with 2 mM IPTG. Two hours after induction with IPTG, ca. 1.0 mmol of crude [3,4,5- $^{13}\text{C}_3$]1-deoxy-D-xylulose (see examples 9 and 10) are added. After three

hours, cells were harvested and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 1.5 ml of 20 mM NaF in D₂O, cooled on ice and sonified 3 x 15 sec. with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the supernatant are recorded directly, without further purification, with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany).

HMQC and HMQC-TOCSY experiments reveal ¹H-¹³C and ¹H-¹H spin systems of [1,3,4-¹³C₃]2C-methyl-D-erythritol 4-phosphate, [1,3,4-¹³C₃]2C-methyl-D-erythritol and [1,3,4-¹³C₅]4-diphosphocytidyl-2C-methyl-D-erythritol (Table 1). The molar ratios of [1,3,4-¹³C₃]2C-methyl-D-erythritol 4-phosphate, [1,3,4-¹³C₃]2C-methyl-D-erythritol and [1,3,4-¹³C₅]4-diphosphocytidyl-2C-methyl-D-erythritol are 1 : 0.6 : 0.9, respectively.

This result indicates that the intracellular amount of CTP required for the synthesis of 4-diphosphocytidyl-2C-methyl-D-erythritol is limiting. Therefore, a modified fermentation protocol was developed (see example 14).

Example 14

Incorporation experiment with recombinant *Escherichia coli* XL1-pBSxylBdxrspDF using [3,4,5-¹³C₃]1-deoxy-D-xylulose

0.1 litre of Luria Bertani (LB) medium containing 18 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harbouring plasmid pBSxylBispDF (see example 4). The cells are grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.5, the culture is induced with 2 mM IPTG. Two hours after induction with IPTG, 10 mg (0.041 mmol) of cytidine and 5 ml of 1 M NaKHPO₄, pH 7.2, and ca. 1 mmol of crude [3,4,5-¹³C₃]1-deoxy-D-xylulose (see examples 9 and 10) are added. After three hours, the cells are harvested and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 µl of 20 mM NaF in D₂O, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the supernatant are recorded directly, without further purification, with a Bruker AVANCE DRX 500 spectrometer. HMQC and HMQC-TOCSY experiments reveal ¹H-¹³C and ¹H-¹H spin systems (Table 1) of

[1,3,4-¹³C₃]2C-methyl-D-erythritol 4-phosphate, [1,3,4-¹³C₃]2C-methyl-D-erythritol and [1,3,4-¹³C₃]4-diphosphocytidyl-2C-methyl-D-erythritol at a molar ratio of approximately 1 : 3.4 : 4.2, respectively. The relative amount of [1,3,4-¹³C₃]4-diphosphocytidyl-2C-methyl-D-erythritol is increased by a factor of 2 as compared to the relative amount in example 13. The intracellular concentration of [1,3,4-¹³C₃]4-diphosphocytidyl-2C-methyl-D-erythritol is estimated as 10 mM by quantitative NMR spectroscopy. The relative high amount of 2C-methyl-D-erythritol indicates that unspecific phosphatases convert intermediary formed 2C-methyl-D-erythritol 4-phosphate into 2C-methyl-D-erythritol. Therefore, a modified fermentation protocol was developed to supply the cells with sufficient amounts of organic phosphates and in order to suppress the activity of phosphatases (see examples 15 to 17).

Example 15

Incorporation experiment with recombinant *Escherichia coli* XL1-pBScyclo using [U-¹³C₅]1-deoxy-D-xylulose

0.2 litre of Luria Bertani (LB) medium containing 36 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harbouring the plasmid pBScyclo (see example 5). The cells are grown in a shaking culture at 37 °C. At an optical density (600 nm) of 1.3, the culture is induced with 2 mM IPTG. Two hours after induction with IPTG, 30 mg (0.12 mmol) of cytidine, 300 mg (0.95 mmol) of DL- α -glycerol 3-phosphate and 10 ml of 1 M NaKHPO₄, pH 7.2, are added. After 30 min, ca. 1 mmol of [U-¹³C₅]1-deoxy-D-xylulose (see example 8 and 10) are added. Aliquots of 25 ml are taken at time intervals of 1 h and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 μ l of 20 mM NaF in D₂O, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the cell free extract are recorded directly, without further purification, with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). ¹³C NMR spectra, as well as HMQC and HMQC-TOCSY spectra established [U-¹³C₅]2C-methyl-D-erythritol 2,4-cyclodiphosphate (Herz *et al.*, 2000) as the only product. Formation of [U-¹³C₅]2C-methyl-D-erythritol 2,4-cyclodiphosphate can be observed 30 min after addition of [U-¹³C₅]1-deoxy-D-xylulose, whereas the maximum yield is observed 5 h after addition of [U-¹³C₅]1-deoxy-D-xylulose. The intracellular concentration of [U-¹³C₅]2C-methyl-D-

erythritol 2,4-cyclodiphosphate is estimated as 20 mM by quantitative NMR spectroscopy. The formation of any other isotope-labelled products, such as [$U-^{13}C_5$]2C-methyl-erythritol is completely suppressed.

Example 16

Incorporation experiment with recombinant *Escherichia coli* XL1-pBScyclo-pACYCgcpE using [$2-^{14}C$]- and [$U-^{13}C_5$]1-deoxy-D-xylulose

0.2 litre of Terrific Broth (TB) medium containing 36 mg of ampicillin and 2.5 mg of chloramphenicol are inoculated with the *E. coli* strain XL1-Blue harbouring the plasmids pBScyclo and pACYCgcpE (see example 5 to 6). The cells are grown in a shaking culture at 37 °C overnight. At an optical density (600 nm) of 4.8 to 5.0, 30 mg (0.1 mmol) of cytidine, 300 mg (0.94 mmol) of DL- α -glycerol 3-phosphate and 10 ml of 1 M NaKHPO₄, pH 7.2, are added. After 30 minutes, a mixture of 2.6 μ mol [$2-^{14}C$]1-deoxy-D-xylulose (15 μ Ci μ mol⁻¹) (Wungsintaweekul et al., 2001) and 1 ml of crude [$U-^{13}C_5$]1-deoxy-D-xylulose (0.02 mmol) (see examples 8 and 10) are added. After 1.5 h, cells are harvested and centrifuged for 10 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in a mixture of 20 mM NaF (2 ml) and methanol (2 ml), cooled on ice and sonified 3 x 15 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4. The suspension is centrifuged at 15,000 rpm for 15 min. The radioactivity of the supernatant is measured by scintillation counting (Beckmann, LS 7800). 10 % of the radioactivity initially added as ¹⁴C labelled 1-deoxy-D-xylulose is detected in the supernatant. Aliquots are analysed by TLC and HPLC, as described in example 19, and the products are purified as described in example 20. On basis of these data, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate were identified as products at a molar ratio of 7 : 3 (see also examples 17 and 18).

Example 17

Incorporation experiment with recombinant *Escherichia coli* XL1-pBScyclo-pACYCgcpE using [$U-^{13}C_5$]- or [$3,4,5-^{13}C_3$]1-deoxy-D-xylulose

0.2 litre of Terrific Broth (TB) medium containing 36 mg of ampicillin and 2.5 mg of

chloramphenicol are inoculated with the *E. coli* strain XL1-Blue harbouring the plasmids pBScyclo and pACYCgcpE. The cells are grown in a shaking culture at 37 °C for overnight. At an optical density (600 nm) of 4.8 - 5.0, 30 mg (0.1 mmol) of cytidine, 300 mg (0.93 mmol) of DL- α -glycerol 3-phosphate and 10 ml of 1 M NaKHPO₄, pH 7.2, are added. After 30 minutes, 3 ml of crude [3,4,5-¹³C₃]- or [U-¹³C₅]1-deoxy-D-xylulose (0.05 mmol) (see examples 8, 9, and 10) are added. Aliquots of 25 ml are taken at time intervals of 1 h and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 μ l of 20 mM NaF in D₂O or in 700 μ l of a mixture of methanol and D₂O (6:4; v/v) containing 10 mM NaF, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the cell free extracts are recorded directly with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). In order to avoid degradation during work-up, the structures of the products are determined by NMR spectroscopy without further purification (see example 18).

Example 18

Structure determination of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate

The ¹H-decoupled ¹³C NMR spectrum using [U-¹³C₅]1-deoxy-D-xylulose as starting material displays 5 ¹³C-¹³C coupled signals belonging to 2C-methyl-D-erythritol 2,4-cyclodiphosphate (Herz et al., 2000) and 5 ¹³C-¹³C coupled signals at 14.7, 64.5, 68.6, 122.7 and 139.5 ppm (Table 2) belonging to an unknown metabolite. The chemical shifts of the unknown metabolite suggest a double bond motif (signals at 122.7 and 139.5 ppm), a methyl group (signal at 14.7 ppm), and two carbon atoms (signals at 64.5 and 68.6 ppm) connected to OR (R=unknown). The three signals accounting for carbon atoms with sp³ hybridisation (14.7, 64.5 and 68.5 ppm) show ¹³C-¹³C coupling to one adjacent ¹³C atom with coupling constants of 40 - 50 Hz (Table 2). The signal at 122.7 ppm shows ¹³C couplings to two adjacent ¹³C neighbours (coupling constants, 74 and 50 Hz), whereas the signal at 141.5 ppm shows ¹³C couplings to three neighboured ¹³C atoms (coupling constants, 74, 43 and 43 Hz). In conjunction with the chemical shift topology, this coupling signature is indicative for a 2-methyl-2-but enyl skeleton. HMQC and HMQC-TOCSY experiments reveal the ¹H NMR chemical shifts (Table 2), as well as ¹³C-¹H and ¹H-¹H spin systems (Table 3). More specifically, the ¹³C NMR signal at 122.7

ppm correlates to a ^1H NMR signal at 5.6 ppm which is in the typical chemical shift range for H-atoms attached to CC double bonds, whereas the signal at 139.5 ppm gives no ^{13}C - ^1H correlations. The signals at 64.5 and 68.6 ppm give ^{13}C - ^1H correlations to ^1H -signals at 4.5 and 3.9 ppm, respectively. The methyl signal at 14.7 ppm correlates to a proton signal at 1.5 ppm. In connection with ^{13}C - ^{13}C coupling patterns (Table 2), as well as with ^1H - ^{13}C long range correlations (HMBC experiment, Table 3), these data establish a 1,4-dihydroxy-2-methyl-2-but enyl system.

Starting from [3,4,5- $^{13}\text{C}_3$]1-deoxy-D-xylulose as feeding material three signals at 64.5, 68.6 and 122.7 ppm accounting for atoms 4, 1 and 3, respectively, of the new product are observed. It can be concluded that the carbon atoms at 1, 3 and 4 of the new product are biogenetically equivalent to the carbon atoms 3, 4 and 5 of [3,4,5- $^{13}\text{C}_3$]1-deoxy-D-xylulose 5-phosphate. This coupling topology is similar to the coupling pattern of 2C-methyl-D-erythritol 4-phosphate (see example 13) confirming that the new compound is derived via 2C-methyl-D-erythritol 4-phosphate.

The C-4 and C-3 ^{13}C NMR signals at 64.5 and 122.7 ppm show ^{13}C - ^{31}P coupling of 5.5 and 8.0 Hz, respectively. These couplings indicate the presence of a phosphate or pyrophosphate group at position 4 of the 2-methyl-2-but enyl skeleton.

In line with this observation, the ^1H -decoupled ^{31}P NMR spectrum of the product displays a doublet at -9.2 (^{31}P - ^{31}P coupling constant, 20.9 Hz) and a double-double-doublet at -10.6 ppm (^{31}P - ^{13}C coupling constants, 5.8 and 7.4 Hz, ^{31}P - ^{31}P coupling constant, 20.9 Hz). Without ^1H -decoupling, the ^{31}P NMR signal at -10.6 ppm is broadened whereas the signal at -9.2 ppm is not affected by ^1H coupling. The chemical shifts as well as the observed coupling pattern confirm the presence of a free diphosphate moiety at position 4.

In summary, all these data establish the structure as 1-hydroxy-2-methyl-2-but enyl 4-diphosphate.

Table 2. NMR data of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate

| Position | Chemical shifts, ppm | | | Coupling constants, Hz | | |
|----------------|----------------------|------------------------------|------------------------------|-------------------------------------|-----------------|---|
| | 1H ^a | ¹³ C ^b | ³¹ P ^c | J _{PC} | J _{PP} | J _{cc} |
| 1 | 3.91 | 68.6 ^{d,e} | | | | 43.0 ^e , 5.5 ^d , 3.5 ^d |
| 2 | | 139.5 ^{d,e} | | | | 74.3 ^e , 43.3 ^e , 43.3 ^e |
| 2-Methyl | 1.51 | 14.7 ^e | | | | 42.2 ^e , 4.0 ^e , 4.0 ^e |
| 3 | 5.57 | 122.7 ^e | | 8.0 ^d | | 73.9 ^e , 49.8 ^d , 4.0 ^d |
| 4 | 4.46 | 64.5 ^{d,e} | | 5.5 ^d | | 49.3 ^d , 5.5 ^d |
| P _β | | | -9.2 | | 20.9 | |
| P _α | | | -10.6 | 5.8 ^d , 7.4 ^d | 20.9 | |

^areferenced to external trimethylsilylpropane sulfonate.^breferenced to external trimethylsilylpropane sulfonate.^creferenced to external 85 % orthophosphoric acid.^dobserved with [1,3,4-¹³C₃]1-hydroxy-2-methyl-2-but enyl 4-diphosphate^eobserved with [U-¹³C₅]1-hydroxy-2-methyl-2-but enyl 4-diphosphateTable 3. Correlation pattern of [1,3,4-¹³C₃]1-hydroxy-2-methyl-2-but enyl 4-diphosphate and of [U-¹³C₅]1-hydroxy-2-methyl-2-but enyl 4-diphosphate

| NMR Correlation pattern | | | |
|-------------------------|-----------------------|-------------------------------------|--|
| Position | HMQC | HMQC-TOCSY | HMBC |
| 1 | 1 ^{a,b} | 1 ^{a,b} | 2-methyl ^a , 2 ^a |
| 2 | | | |
| 2-methyl | 2-methyl ^b | 2-methyl ^b | |
| 3 | 3 ^{a,b} | 3 ^{a,b} , 4 ^{a,b} | 2-methyl ^a , 1 ^a |
| 4 | 4 ^{a,b} | 4 ^{a,b} , 3 ^{a,b} | |

^aobserved with [1,3,4-¹³C₃]1-hydroxy-2-methyl-2-but enyl 4-diphosphate^bobserved with [U-¹³C₅]1-hydroxy-2-methyl-2-but enyl 4-diphosphate

Example 19

Detection of phosphorylated metabolites of the mevalonate-independent pathway

Method A) By a TLC method

Aliquots (10 µl) of the cell-free extracts from recombinant cells prepared as described above (see example 16) are spotted on a Polygram® SIL NH-R thin layer plate (Macherey-Nagel, Düren, Germany). The TLC plate is then developed in a solvent system of n-propanol: ethyl acetate: water; 6:1:3 (v/v/v). The running time is about 4 h. The radio chromatogram is monitored and evaluated by a Phosphor Imager (Storm 860, Molecular Dynamics, USA). The R_f-values of the compounds under study are shown in Table 4.

Table 4: R_f-values of precursors and intermediates of the mevalonate-independent terpenoid pathway

| Chemical compound | R _f -value |
|---|-----------------------|
| 1-deoxy-D-xylulose | 0.80 |
| 1-deoxy-D-xylulose 5-phosphate | 0.5 |
| 2C-methyl-D-erythritol 4-phosphate | 0.42 |
| 4-diphosphocytidyl-2C-methyl-D-erythritol | 0.33 |
| 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate | 0.27 |
| 2C-methyl-D-erythritol 2,4-cyclodiphosphate | 0.47 |
| 1-hydroxy-2-methyl-2-but enyl 4-diphosphate | 0.17 |

Method B) By a HPLC method

Aliquots (100 µl) of the cell-free extracts from recombinant cells prepared as described above (see example 16), are analyzed by HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm, particle size 5 µm, CS-Chromatographic Service GmbH, Langerwehe, Germany) that has been equilibrated for 15 min with 10 mM tetrabutylammonium hydrogensulfate (TBAS), pH 6.0, at a flow rate of 0.75 ml min⁻¹. After injection of the sample, the column is developed for 20 min with 10 mM TBAS, then for 60 min with a linear gradient of 0 - 42 % (v/v)

methanol in 10 mM TBAS. The effluent is monitored by a continuous-flow radio detector (Beta-RAM, Biostep GmbH, Jahnsdorf, Germany). The retention volumes of the compounds under study are shown in Table 5.

Table 5: Retention volumes of precursors and intermediates of the mevalonate-independent terpenoid pathway

| Chemical compound | Retention volume [ml] |
|---|-----------------------|
| 1-deoxy-D-xylulose | 6.0 |
| 1-deoxy-D-xylulose 5-phosphate | 15 |
| 2C-methyl-D-erythritol 4-phosphate | 13.5 |
| 4-diphosphocytidyl-2C-methyl-D-erythritol | 30.8 |
| 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate | 41.3 |
| 2C-methyl-D-erythritol 2,4-cyclodiphosphate | 31.5 |
| 1-hydroxy-2-methyl-2-but enyl 4-diphosphate | 42.8 |

Example 20

Purification of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate

The crude cell free extract obtained from the feeding experiment with recombinant *Escherichia coli* XL1-pBScyclo-pACYCgcpE using [$2\text{-}^{14}\text{C}$]- and [$\text{U-}^{13}\text{C}_5$]-1-deoxy-D-xylulose (see example 16) is lyophilized. The residue is dissolved in 600 μl of water and centrifuged for 10 min at 14,000 ppm. Aliquots of 90 μl are applied on a column of Nucleosil 10 SB (4.6 x 250 mm, Macherey & Nagel, Düren, Germany) which is developed with a linear gradient of 0.1 - 0.25 M ammonium formate in 70 ml at a flow rate of 2 ml min^{-1} . The retention volumes for 2C-methyl-D-erythritol-2,4-cyclodiphosphate and 1-hydroxy-2-methyl-2-but enyl 4-diphosphate are 25 and 44 ml, respectively. Fractions are collected and lyophilized. NMR data of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate are identical with the data shown in example 18, Table 2.

Example 21

Construction of a vector carrying the *xyIB*, *dxr*, *ispD*, *ispE*, *ispF* and *ispG* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase, CDP-

ME synthase, CDP-ME kinase cMEPP synthase and 1-hydroxy-2-methyl-2-but enyl 4-diphosphate synthase

The *E. coli* ORF *ispG* (accession no. gb AE000338) from base pair (bp) position 372 to 1204 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GCGGGAGACCGCGGGAGGAGAAATTAAACCATGCATAACCAGGCTCCAATTG-3', 10 pmol of the primer 5'-CGCTTCCCAGCGGCCGCTTATTTTCAACCTGCTGAACG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.4 μ g of the vector pBScyclo (Example 5) and 0.8 μ g of the purified PCR product are digested with SacII and NotI in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 18 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pBScyclogcpE. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScyclogcpE is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScyclogcpE is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000338). The DNA sequence of

the vector construct pBScyclogcpE is shown in Annex H.

Example 22

Construction of a vector carrying the *xylB*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG* and *lytB* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase, CDP-ME synthase, CDP-ME kinase, cMEPP synthase, 1-hydroxy-2-methyl-2-but enyl 4-diphosphate synthase and LytB

The *E. coli* ORF *lytB* (accession no. gb AE005179) from base pair (bp) position 7504 to 8454 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-AAATCGGAGCTCGAGGAGAAATTAAACCATGCAGATCCTGTTGGCC-3', 10 pmol of the primer 5'-GCTGCTCCGCGGTTAACGACTTCACGAATATCG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.3 µg of the vector pBScyclogcpE (Example 21) and 0.7 µg of the purified PCR product are digested with SacI and SacII in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 16 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pBScyclogcpElytB. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScyclogcpElytB is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScyclogcpElytB is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE005179).

Example 23

Incorporation experiment with recombinant *Escherichia coli* XL1-pBScyclogcpE using [$U-^{13}C_5$]1-deoxy-D-xylulose

0.1 litre of Terrific Broth (TB) medium containing 18 mg of ampicillin are inoculated with *E. coli* strain XI1-Blue harbouring the plasmid pBScyclogcpE. The cells are grown in a shaking culture at 37 °C overnight. At an optical density (600 nm) of 4.8 – 5.0, 30 mg (0.1 mmol) of cytidine are added. A solution containing 1.2 g of lithium lactate (12.5 mmol), 6 ml of crude [$U-^{13}C_5$]1-deoxy-D-xylulose (0.05 mmol) (see examples 8, 9 and 10) in 0.1 M Tris hydrochloride (pH=7.5) at a final volume of 30 ml are added continuously within 2 hours. Aliquots of 25 ml are taken at time intervals of 1 h and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 µl of 20 mM NaF in D₂O or in 700 µl of a mixture of methanol and D₂O (6:4, v/v) containing 10 mM NaF, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the cell free extracts are recorded directly with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). In order to avoid degradation during work-up, the structures of the products are determined by NMR spectroscopy without further purification.

The relative amount of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate could be raised by a factor of approximately 2-3 by the addition of lithium lactate to the medium.

Example 24

Preparation of (*E*)-1-hydroxy-2-methyl-2-butenyl diphosphate triammonium salt (8)

General. Chemicals are obtained from Acros Organics (Fisher Scientific GmbH, Schwerte, Germany), SIGMA-ALDRICH (Deisenhofen, Germany), MERCK (Darmstadt, Germany) and used without further purification. Solvents are used distilled and/or dried. Chromatography is performed on silica gel 60 (230-400 mesh, Fluka Riedel-de Haen, Taufkirchen, Germany), DOWEX 50 WX8 (200-400 mesh, SERVA, Heidelberg, Germany), and Cellulose (Avicel, Cellulose mikrokristallin, Merck, Darmstadt, Germany). TLC is performed on silica gel 60 F_{254} plastic sheets (MERCK) or cellulose F plastic sheets (MERCK), detection by anisaldehyde solution (anisaldehyde:H₂SO₄:HAc 0.5:1:50 v/v/v). NMR-spectra are recorded on BRUKER AMX 400, DRX 500, and AC 250 spectrometer at room temperature.

Acetonyl tetrahydropyranyl ether (12) (Hagiwara *et al.*, 1984).

A mixture of 339 mg (1.35 mmol) of pyridinium-toluene-4-sulfonate, 9.35 ml (10.0 g, 0.135 mol) of hydroxyacetone, and 24.7 ml (22.7 g, 0.270 mol) of 3,4-dihydro-2H-pyran is stirred at room temperature for 2.5 h. Residual 3,4-dihydro-2H-pyran is removed under reduced pressure. The crude mixture is purified by FC on silicagel (hexanes/acetone 4:1, 6.5 × 20 cm) to yield 18.7 g (0.118 mol, 88%) of a colorless liquid.

¹H NMR (CDCl₃, 500 MHz) δ 4.62 (t, *J* = 3.6 Hz, 1H), 4.22 (d, *J* = 17.3 Hz, 1H), 4.09 (d, *J* = 17.3 Hz, 1H), 3.83-3.79 (m, 1H), 3.51-3.47 (m, 1H), 2.15 (s, 3H), 1.87-1.49 (m, 6H); ¹³C NMR (CDCl₃, 126 MHz) δ 206.7, 98.7, 72.3, 62.3, 30.2, 26.5, 25.2, 19.2; MS (Cl, isobutane) *m/z* 159 [M + 1]⁺.

(E,Z)-Ethyl-2-methyl-1-tetrahydropyranyloxy-but-2-enoate (13) (Watanabe *et al.*, 1996).

33.0 g (94.8 mmol) of (ethoxycarbonylmethylen)-triphenylphosphorane are dissolved in 500 ml of dry toluene under nitrogen atmosphere at room temperature. Then, 10.0 g (63.2 mmol) of acetonyl tetrahydropyranyl ether **12** are added and the mixture is heated to reflux. After 39 h at this temperature the solvent is evaporated *under reduced pressure* to yield an orange oil. Major amounts of triphenylphosphinoxide are precipitated by the addition of 100 ml hexanes/acetone 9:1. After filtration the filtrate is concentrated and another 100 ml of hexanes/acetone 9:1 are added. The solid is filtered off and the solvent removed to yield 18 g of an orange oil that is purified by FC on silicagel (hexanes/acetone 9:1, 6.5 × 28 cm) to yield 12.9 g (56.5 mmol, 89%) of a mixture of **(E)-13/(Z)-13 = 5:1**.

(E)-13. ¹H NMR (CDCl₃, 500 MHz) δ 5.96 (q, *J* = 1.4 Hz, 1H), 4.62 (t, *J* = 3.5 Hz, 1H), 4.20 (dd, *J* = 15.5 Hz, 1.3 Hz, 1H), 4.14 (q, *J* = 7.1 Hz, 2H), 3.93 (dd, *J* = 15.6, 1.3 Hz, 1H), 3.84-

3.79 (m, 1H), 3.52-3.48 (m, 1H), 2.08 (d, J = 1.4 Hz, 3H), 1.88-1.50 (m, 6H), 1.26 (t, J = 7.1 Hz, 3H); ^{13}C NMR (CDCl₃, 126 MHz) δ 166.8, 154.7, 114.5, 98.0, 70.6, 62.0, 59.7, 30.3, 25.3, 19.1, 15.9, 14.3; MS (Cl, isobutane) *m/z* 229 [M + 1]⁺.

(Z)-(13). ^1H NMR (CDCl₃, 500 MHz) δ 5.71 (q, J = 1.4 Hz, 1H), 4.60 (t, J = 3.6 Hz, 1H), 4.20 (dd, J = 15.5 Hz, 1.3 Hz, 1H), 4.11 (q, J = 7.1 Hz, 2H), 3.93 (dd, J = 15.6, 1.3 Hz, 1H), 3.84-3.79 (m, 1H), 3.52-3.48 (m, 1H), 1.97 (d, J = 1.4 Hz, 3H), 1.88-1.50 (m, 6H), 1.24 (t, J = 7.1 Hz, 3H); ^{13}C NMR (CDCl₃, 126 MHz) δ 165.9, 156.8, 116.9, 98.7, 66.5, 62.3, 59.8, 30.6, 25.3, 21.9, 19.5, 14.3; MS (Cl, isobutane) *m/z* 229 [M + 1]⁺.

(E,Z)-2-Methyl 1-tetrahydropyranloxy-but-2-ene-4-ol (14) (Watanabe *et al.*, 1996).

A solution of ester **13** (8.73 g, 38.2 mmol) in 100 ml of dry CH₂Cl₂ is cooled to -78 °C. Then, 91.8 ml (91.8 mmol) of 1.0 M DIBAH in hexanes are added slowly under an atmosphere of nitrogen. The resulting solution is stirred for 3 h at -78 °C before the reaction is quenched by the addition of 1.5 ml of 1 M NaOH. After warming to room temperature the solvent is removed *under reduced pressure*. The resulting gummy residue is widely dissolved by adding twice 100 ml of MeOH. The resulting mixture is passed through a column of SiO₂, evaporated from the solvent and then loaded on a column of SiO₂/Na₂SO₄ that is purged with 1400 ml of MeOH. Evaporation of the solvent gives 9.5 g of a colorless liquid that is purified by FC on silica gel (hexanes/acetone 1:3, 6.5 × 16 cm) to yield 6.98 g (37.4 mmol, 98%) of a colorless liquid **(E)-14/(Z)-14 = 6:1**.

(E)-(14). ^1H NMR (CDCl₃, 500 MHz) δ 5.68 (tq, J = 6.6, 1.3 Hz, 1H), 4.60 (t, J = 3.6 Hz, 1H), 4.20 (d, J = 6.8 Hz, 2H), 4.12 (d, J = 12.0 Hz, 1H), 3.87-3.82 (m, 1H), 3.85 (d, J = 12.5 Hz, 1H), 3.52-3.48 (m, 1H), 1.86-1.48 (m, 6H), 1.69 (s, 3H); ^{13}C NMR (CDCl₃, 126 MHz) δ 135.7, 125.4, 97.8, 71.9, 62.1, 59.1, 30.5, 25.4, 19.4, 14.1; MS (Cl, isobutane) *m/z* 169 [M - H₂O + 1]⁺.

(Z)-(14). ^1H NMR (CDCl₃, 500 MHz) δ 5.64 (tq, J = 6.6, 1.3 Hz, 1H), 4.63 (t, J = 3.3 Hz, 1H), 4.20 (d, J = 6.8 Hz, 2H), 4.15 (d, J = 11.8 Hz, 1H), 3.87-3.82 (m, 1H), 3.83 (d, J = 11.3 Hz, 1H), 3.52-3.48 (m, 1H), 1.86-1.48 (m, 6H), 1.79 (s, 3H); ^{13}C NMR (CDCl₃, 126 MHz) δ 136.2, 128.6, 96.6, 65.1, 61.8, 58.1, 30.3, 25.3, 21.9, 19.0; MS (Cl, isobutane) *m/z* 169 [M-H₂O+1]⁺.

(E,Z)-4-Chloro-2-methyl 1-tetrahydropyranloxy-but-2-en (15) (Hwang *et al.*, 1984).

To a solution of alcohol **14** (1.00 g, 5.37 mmol) in 10 ml of dry CH₂Cl₂ are added 918 mg (7.52 mmol) of DMAP in 10 ml of dry CH₂Cl₂ and 1.23 g (6.44 mmol) of *p*-TsCl in 10 ml of dry

CH_2Cl_2 . The resulting solution is stirred at room temperature for 1 h. After evaporation of the solvent *under reduced pressure* the residue is purified by FC on silica gel (CH_2Cl_2 , 5 × 20 cm) to obtain 693 mg (3.39 mmol, 63%) of a colorless liquid (**E**)-15/(**Z**)-15 = 6:1.

(E)-(15). ^1H NMR (CDCl_3 , 500 MHz) δ 5.77 (tq, J = 8.0, 1.5 Hz, 1H), 4.64 (t, J = 3.6 Hz, 1H), 4.18 (d, J = 12.8 Hz, 1H), 4.15 (d, J = 8.0 Hz, 2H), 3.92 (d, J = 12.8 Hz, 1H), 3.90-3.86 (m, 1H), 3.59-3.52 (m, 1H), 1.92-1.52 (m, 6H), 1.77 (s, 3H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 138.6, 121.7, 97.8, 71.3, 62.1, 40.2, 30.5, 25.4, 19.3, 13.9; MS (Cl, isobutane) m/z 205 [M + 1]⁺.

(Z)-(15). ^1H NMR (CDCl_3 , 500 MHz) δ 5.65 (t, J = 8.1 Hz, 1H), 4.61 (t, J = 3.6 Hz, 1H), 4.18 (d, J = 12.8 Hz, 1H), 4.15 (d, J = 8.0 Hz, 2H), 3.92 (d, J = 12.8 Hz, 1H), 3.90-3.86 (m, 1H), 3.59-3.52 (m, 1H), 1.92-1.52 (m, 6H), 1.86 (s, 3H); ^{13}C NMR (CDCl_3 , 126 MHz) δ 138.3, 124.6, 97.5, 64.7, 62.2, 40.1, 30.5, 25.4, 21.8, 19.4; MS (Cl, isobutane) m/z 205 [M + 1]⁺.

(E,Z)-2-Methyl 1-tetrahydropyranloxy-but-2-enyl diphosphate triammonium salt (16)
(Davisson *et al.*, 1986).

To a solution of chloride **15** (260 mg, 1.27 mmol) in 1.3 ml of MeCN a solution of 1.38 g (1.52 mmol) tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in 3.0 ml of MeCN is added slowly at room temperature, obtaining an orange-red solution. The reaction is followed by ^1H -NMR, taking advantage of the up field shift of the multiplet of H-3. After 2 h the reaction is finished and the solvent removed *under reduced pressure*. The orange oil is dissolved in 2.5 ml of H_2O and passed through a column of DOWEX 50 WX8 (2.5 × 3 cm) cation-exchange resin (NH_4^+ form) that has been equilibrated with two column volumes (40 ml) of 25 mM NH_4HCO_3 . The column is eluted with 60 ml of 25 mM NH_4HCO_3 . The resulting solution is lyophilized, dissolved in 5 ml of isopropanol/100 mM NH_4HCO_3 1:1 and loaded on a cellulose column (2 × 18 cm) that is eluted by isopropanol/100 mM NH_4HCO_3 1:1. The effluent is lyophilized obtaining 495 mg (1.25 mmol, 98%) of (**E**)-16/(**Z**)-16 = 6:1 as a white solid.

(E)-(16). ^1H NMR (D_2O , 500 MHz) δ 5.52 (tq, J = 6.8 Hz, 1H), 4.65 (s, 1H), 4.34 (t, J = 7.0 Hz, 2H), 3.98 (d, J = 12.3 Hz, 1H), 3.84 (d, J = 12.1 Hz, 1H), 3.74-3.70 (m, 1H), 3.42-3.38 (m, 1H), 1.61-1.57 (m, 2H), 1.54 (s, 3H), 1.40-1.32 (m, 4H); ^{13}C NMR (D_2O , 126 MHz) δ 136.4, 123.9 (dd, J = 8.0, 2.3 Hz), 98.5, 72.5, 63.2, 62.2 (d, J = 5.3 Hz), 29.9, 24.5, 19.0, 13.4; ^{31}P NMR (D_2O , 101 MHz) δ -5.62 (d, J = 20.9 Hz), -7.57 (d, J = 20.8 Hz).

(Z)-(16). ^1H NMR (D_2O , 500 MHz) δ 5.52 (t, J = 6.8, 1H), 4.65 (s, 1H), 4.31 (t, J = 7.1 Hz, 2H), 3.98 (d, J = 12.3 Hz, 1H), 3.84 (d, J = 12.1 Hz, 1H), 3.74-3.70 (m, 1H), 3.42-3.38 (m, 1H), 1.64

(s, 3H), 1.61-1.57 (m, 2H), 1.40-1.32 (m, 4H); ^{13}C NMR (D_2O , 126 MHz) δ 136.3, 125.8 (d, J = 8.6 Hz), 98.6, 72.5, 63.2, 61.8 (d, J = 5.1 Hz), 29.9, 24.5, 20.8, 19.0; ^{31}P NMR (D_2O , 101 MHz) δ -5.69 (d, J = 20.8 Hz), -7.68 (d, J = 20.8 Hz).

(E,Z)-1-Hydroxy-2-methyl-but-2-enyl diphosphate triammonium salt (8) (Davisson *et al.*, 1986).

268 mg (0.675 mmol) of protected pyrophosphate **16** are dissolved in 2.0 ml of D_2O and the pH is adjusted to 1 by addition of 40 μl of 37% DCI in D_2O . After 1 min at this pH the solution is neutralized by addition of 40 μl of 40% NaOD in D_2O and an ^1H NMR is measured that demonstrated 50% deprotection. The procedure is repeated until deprotection is finished and just small amounts of decomposition product are formed to get in total 7 min at pH 1. Purification is performed by loading the neutral solution that is diluted by addition of 2 ml of isopropanol/100 mM NH_4HCO_3 1:1 on a cellulose column (isopropanol/100 mM NH_4HCO_3 1:1, 2 \times 10.5 cm) to yield 193 mg (0.616 mmol, 91%) of a white solid of (E)-**8**/(Z)-**8** = 7:1.

(E)-8**.** ^1H NMR (D_2O , 500 MHz) δ 5.51 (tq, J = 6.8, 1.2 Hz, 1H), 4.41 (t, J = 7.2 Hz, 2H), 3.90 (s, 2H), 1.59 (s, 3H); ^{13}C NMR (D_2O , 126 MHz) δ 139.8, 120.6 (d, J = 7.7 Hz), 66.5, 62.4 (d, J = 5.3 Hz), 13.2; ^{31}P NMR (D_2O , 101 MHz) δ -4.48 (d, J = 20.8 Hz), -7.06 (d, J = 20.8 Hz).

(Z)-8**.** ^1H NMR (D_2O , 500 MHz) δ 5.49 (tm, J = 6.8 Hz, 1H), 4.41 (t, J = 7.3 Hz, 2H), 4.03 (s, 2H), 1.70 (s, 3H); ^{13}C NMR (D_2O , 126 MHz) δ 139.8, 123.5 (d, J = 7.7 Hz), 61.7 (d, J = 5.1 Hz), 59.9, 20.6; ^{31}P NMR (D_2O , 101 MHz) δ -4.48 (d, J = 20.8 Hz), -7.06 (d, J = 20.8 Hz).

Reagents and conditions (steps (a) to (f) in Fig. 4: 1): (a) DHP, PPTS, 25 °C (2.5 h); (b) $\text{Ph}_3\text{PCHCO}_2\text{Et}$, toluene, reflux (39 h); (c) (1) DIBAH, CH_2Cl_2 , -78 °C (3 h), (2) 1 M NaOH/ H_2O ; (d) *p*-TsCl, DMAP, CH_2Cl_2 , 25 °C (1 h); (e) $((\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2)_4\text{N})_3\text{HP}_2\text{O}_7$, MeCN, 25 °C (2 h); (f), HCl/ H_2O pH 1, 25 °C (7 min).

Example 25

Identification of (E)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate

The structure of the GcpE product is further analyzed by comparison with the chemical shifts of a synthetic sample of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate.

For this purpose, [2-¹⁴C]1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (0.36 µCi) is added to a cell extract obtained from bioengineered *Escherichia coli* cells endowed with artificial gene constructs expressing *xyB*, *ispC*, *ispD*, *ispE*, *ispF* and *gcpE* gene which are supplied with [U -¹³C₅]-1-deoxy-D-xylulose (see example 16). The supernatant of the cell extract is purified by HPLC (Nucleosil 5 SB, 7.5 × 250 mm, developed with a gradient of 100 mM to 250 mM NH₄HCOO, flow rate 2 ml/min, 35 min). The product is eluted at 23 min and collected. After lyophilization the residue is dissolved in D₂O (pH 6) and subjected to ¹H NMR analysis (Figure 3-A).

Then, 40 µl of a solution of synthetically prepared (*E,Z*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate (*E/Z* = 7:1) (D₂O, pH 7) are added to the NMR sample and again analyzed by ¹H NMR spectroscopy (Figure 3-B). On the one hand, as shown in Figure 3-B, signals accounting for (*E*)-1-hydroxy-2-methyl-2-but enyl are selectively increased, providing evidence that the biologically produced structure is identical with the synthetically produced one, i.e. the (*E*)-isomer. On the other hand, the minor (*Z*)-isomer raises without any correlation to signals of the biologically afforded product. Figure 3-C shows the same effects after addition of another 40 µl of solution of the synthetically prepared (*E,Z*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate.

Example 26

Incorporation of (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate into the lipid soluble fraction of *Capsicum annuum* chromoplasts.

Chromoplasts are isolated by a slight modification of a method described by Camara (Camara, 1985; Camara, 1993). Pericarp of red pepper (650 g) is homogenized at 4 °C in 600 ml of 50 mM Hepes, pH 8.0, containing 1 mM DTE, 1 mM EDTA and 0.4 M sucrose (buffer A). The suspension is filtered through four layers of nylon cloth (50 µm) and centrifuged (10 min, 4,500 rpm, GSA rotor) to obtain a pellet of crude chromoplasts which is homogenized in 200 ml of buffer A. The suspension is centrifuged (10 min, 4,500 rpm, GSA rotor). The pellet is homogenized and resuspended in 3 ml of 50 mM Hepes, pH 7.6, containing 1 mM DTE. The suspension is filtered through one layer of nylon cloth (50 µm).

Reaction mixtures contain 100 mM Hepes, pH 7.6, 2 mM MnCl₂, 10 mM MgCl₂, 5 mM NaF, 2 mM NADP⁺, 1 mM NADPH, 6 mM ATP, 20 µM FAD and 2 mg of chromoplasts. 8.8 nmol of [2-¹⁴C]2C-methyl-D-erythritol 2,4-cyclodiphosphate, [2-¹⁴C]1-hydroxy-2-methyl-2-(*E*)-butenyl diphosphate or [2-¹⁴C]isopentenyl diphosphate (specific concentrations 15.8 µCi/µmol) are

added and the mixtures are incubated at 30 °C overnight. The reaction is terminated by methylene chloride extraction. The organic phase is concentrated under a stream of nitrogen. Aliquots are spotted on silica gel plates (Polygram SIL-G, UV254, Macherey-Nagel, Düren, Germany). The plates are developed with hexane : ether = 6: 1 (system I) and/or hexane : toluene = 9 : 1 (system II), respectively. The chromatograms are monitored with a phosphor imager (Storm 860, Molecular dynamics, Sunnyvale, CA, USA). The R_f -values of geranylgeraniol and the carotene fraction in system I are 0.35 and 0.9, respectively. The R_f -values of β -carotene, phytoene and phytofluene in system II are 0.65, 0.60 and 0.55, respectively.

The evaluation of the chromatogramms show that radioactivity can be efficiently diverted from 1-hydroxy-2-methyl-2-(*E*)-butenyl diphosphate into the geranylgeraniol, β -carotene, phytoene and phytofluene fractions of *C. annuum* chromoplasts establishing 1-hydroxy-2-methyl-2-(*E*)-butenyl diphosphate as a real intermediate of the non-mevalonate pathway downstream from 2C-methyl-D-erythritol 2,4-cyclodiphosphate and upstream from isopentenyl diphosphate.

Example 27

Construction of a vector carrying the *ispG* (*gcpE*) and *ispH* (*lytB*) genes of *Escherichia coli* capable for transcription and expression thereof

The *E. coli* ORF *ispH* (*lytB*) (accession no. gb AE000113) from base pair (bp) position 5618 to 6568 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GCTTGCCTCGACGAGGGAGAAATTAAACCATGCAGATCCTGTTGGCCACC-3', 10 pmol of the primer 5'-GCTGCTCGGCCGTTAACGACTTCACGAATATCG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.4 µg of the vector pACYC184 (Chang and Cohen 1978, NEB) and 0.7 µg of the purified PCR product are digested with *Sall* and *Eagl* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 18 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYClytB. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYClytB is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYClytB is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prismä Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000113).

The *E. coli* ORF *ispG* (*gcpE*) (accession no. gb AE000338) from base pair (bp) position 372 to 1204 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5' - CGTACCGGATCCGAGGGAGAAATTAAACCATGCATAACCAGGGCTCCAATT-3', 10 pmol of the primer 5'-CCCATCGTCGACTTATTTTCAACCTGCTGAACGTC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.0 µg of the vector pACYClytB and 0.9 µg of the purified PCR product are digested with *Bam*HI and *Sall* in order to produce DNA fragments with overlapping ends. The restriction

mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 23 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYClytBgcpE. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYClytBgcpE is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYClytBgcpE is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000338).

The DNA sequence of the vector construct pACYClytBgcpE is shown in Annex I.

The DNA and corresponding amino acid sequence of *ispH* (*lytB*) from *Escherichia coli* is shown in Annex J.

Example 28

Construction of a vector carrying the *xytB*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG* and *ispH* genes of *Escherichia coli* capable for transcription and expression of D-xylulokinase, DXP reductoisomerase, CDP-ME synthase, CDP-ME kinase cMEPP synthase, 1-hydroxy-2-methyl-2-but enyl 4-diphosphate synthase and IPP/DMAPP synthase

The *E. coli* ORFs *ispG* (formerly *gcpE*) and *ispH* (formerly *lytB*) are amplified by PCR using the plasmid pACYClytBgcpE (see example 27) as template. The reaction mixture contains 10 pmol o f t h e p r i m e r 5' -
GCGGGAGACCGCGGGAGGAGAAATTAAACCATGCATAACCAGGCTCCAATTCAACG', 10 pmol of the primer 5'-AGGCTGGCGGCCGCTTAATCGACTTCACGAATATCG-3', 2 ng of pACYCgcpElytB DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 150 sec at 72 °C followed. After further incubation for 20 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

1.7 µg of the vector pBScyclo (Example 5) and 1.3 µg of the purified PCR product are digested with *Sac*II and *Not*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

22 ng of the purified vector DNA and 19 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pBScyclogcpElytB2. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pBScyclogcpElytB2 is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pBScyclogcpElytB2 is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. The DNA sequence of the vector construct pBScyclogcpElytB2 is shown in Annex K.

Example 29

Incorporation experiment with recombinant *Escherichia coli* XL1-pBScyclogcpElytB2 using [$^{\text{U}}\text{-}{}^{13}\text{C}_5$]1-deoxy-D-xylulose

0.1 litre of Terrific Broth (TB) medium containing 18 mg of ampicillin are inoculated with *E. coli* strain XI1-Blue harbouring the plasmid pBScyclogcpElytB2. The cells are grown in a shaking culture at 37 °C for overnight. At an optical density (600 nm) of 1.3-1.7 a solution containing 2.4 g of lithium lactate (25 mmol), 10 ml of crude [$^{\text{U}}\text{-}{}^{13}\text{C}_5$]1-deoxy-D-xylulose (0.05 mmol) (see example 8) at a final volume of 30 ml (pH=7.4) are added continuously within 2 hours. Aliquots

of 40 ml are taken at time intervals of 30 minutes and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 ml of a mixture of methanol and D₂O (6:4, v/v) containing 10 mM NaF, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the cell free extracts are recorded directly with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). In order to avoid degradation during work-up, the structures of the products are determined by NMR spectroscopy without further purification.

Example 30

Structure determination of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)

The ¹H-decoupled ¹³C NMR spectrum using [U-¹³C₅]1-deoxy-D-xylulose as starting material (see examples 8 and 30) displays five intense ¹C-¹³C coupled signals belonging to 2C-methyl-D-erythritol 2,4-cyclodiphosphate (Herz et al., 2000) and five ¹³C-¹³C coupled signals with low intensities belonging to 1-hydroxy-2-methyl-2-butanyl 4-diphosphate (see example 18) (100:3 ratio for the 2-methyl ¹³C NMR signal intensities of 2C-methyl-D-erythritol 2,4-cyclodiphosphate and 1-hydroxy-2-methyl-2-butanyl 4-diphosphate, respectively).

In addition a set of , five ¹³C-¹³C coupled signals at 21.6 (doublet), 37.8 (triplet), 64.1 (doublet), 111.6 (doublet), and 143.3 ppm (doublet of triplets) (unknown metabolite A) accompanied by signals at 21.1 (doublet), 39.6 (triplet), 59.3 (doublet), 111.8 (doublet), and 143.2 ppm (doublet of triplets) (unknown metabolite B) is detected. The ratio of the 2-methyl signal of 2C-methyl-D-erythritol 2,4-cyclodiphosphate and the putative methyl signals of the unknown compounds at 21.6 ppm (metabolite A) and 21.1 ppm (metabolite B) is 100:24:4, respectively.

Moreover, ¹³C coupled signals with low intensities belonging to another unknown compound (metabolite C) at 17.1 (doublet), 24.9 (doublet), 62.7 (doublet), 119.6 (double-doublet) and 139.4 ppm (multiplet) are detected. The ratio of the intensities of the putative methyl signals at 21.6 (metabolite A), 17.1 and 24.9 (metabolite C) is 100:13:13, respectively.

The ³¹P NMR spectrum of the reaction mixture is characterized by intense signals for 2C-

methyl-D-erythritol 2,4-cyclodiphosphate (Herz et al., 2000). Furthermore, $^{31}\text{P}^{31}\text{P}$ coupled broadened signals are observed at a chemical shift range typical for organic diphosphates (-6 to -13 ppm, $^{31}\text{P}^{31}\text{P}$ coupling constants, 20 Hz).

Metabolite A:

The signals of metabolite A at 111.6 and 143.3 ppm are conducive of a double bond motif, and the signals at 64.1, 37.8 and 21.6 ppm reflect three aliphatic carbon atoms one of which (signal at 64.1 ppm) appears to be connected to OH or OR (R=unknown).

Additional information about the structure of the unknown metabolite A can be gleaned from the ^{13}C coupling pattern. Three of the ^{13}C NMR signals (21.6, 64.1 and 111.6 ppm) are split into doublets indicating three ^{13}C atoms each connected to only one ^{13}C -labelled neighbour, one signal (37.8 ppm) displays a pseudo-triplet signature indicating a ^{13}C atom with two adjacent ^{13}C atoms, and one signal (143.3 ppm) is split into a doublet of triplets indicating a ^{13}C atom with three ^{13}C connections. In conjunction with the chemical shifts, this connectivity pattern establish metabolite A as an isopentenyl derivative.

The complex signature for the signal at 143.3 ppm deserves a more detailed analysis. The large coupling (71 Hz) is typical for $^{13}\text{C}^{13}\text{C}$ couplings between carbon atoms involved in carbon-carbon double bonds. A 71 Hz coupling is also found for the doublet signal at 111.6 ppm representing the second carbon of the double bond. Due to the coupling pattern and the chemical shifts the presence of an exo-methylene function is obvious. The two additional ^{13}C couplings found in the triplet substructure of the signal at 143.3 ppm are both 41 Hz, and establish the respective carbon as the branching point of the structure.

HMQC experiments reveal the ^1H NMR chemical shifts, as well as ^{13}C - ^1H and ^{1}H - ^1H spin systems. More specifically, the ^{13}C NMR signal at 111.6 ppm correlates to a ^1H NMR signal at 4.73 ppm, whereas the signal at 143.3 ppm gives no ^{13}C - ^1H correlation. The signals at 64.1, 37.8, and 21.6 ppm give ^{13}C - ^1H correlations to ^1H -signals at 4.00, 2.31, and 1.68 ppm, respectively. As shown by HMQC-TOCSY experiments, the proton signals at 2.31 and 4.00 are coupled, whereas the signals at 4.73 and 1.68 ppm are found as singlets in the HMQC-TOCSY experiment. The observed ^1H NMR chemical shifts in combination with the coupling patterns demonstrate that metabolite A is an isopentenyl derivative with a single bonded heteroatom (most plausibly O) at position 1.

The ^{31}C and ^1H chemical shifts of an authentic sample of isopentenyl diphosphate (IPP, measured in the same solvent mixture) are identical to the chemical shifts assigned to

metabolite A. Thus, metabolite A is identified as [$^{\text{U}}\text{-}{}^{\text{13}}\text{C}_5$]IPP.

Metabolite B:

As noted above, the coupling and correlation pattern of metabolite B observed in the ${}^{\text{13}}\text{C}$ NMR signals, as well as in the HMQC and HMQC-TOCSY spectra, is virtually the same as for metabolite A (IPP) suggesting that the carbon connectivities of metabolite B and IPP are identical. As the most significant difference between the NMR data of metabolite B and IPP the ${}^{\text{13}}\text{C}$ NMR chemical shift of one doublet signal for metabolite B (59.3 ppm) corresponding to the C-1 signal of IPP (64.1 ppm) is upfield shifted by 4.9 ppm. This suggests that a phosphate moiety is missing at C-1 in metabolite B. Therefore, metabolite B is assigned as [$^{\text{U}}\text{-}{}^{\text{13}}\text{C}_5$]isopentene-1-ol. Presumably, isopentene-1-ol is formed from IPP by the catalytic action of pyrophosphatases and phosphatases present in the experimental system.

Metabolite C:

As described above for metabolite A (IPP), the structure of metabolite C is assigned by NMR analysis. The ${}^{\text{13}}\text{C}$ coupling pattern of the signals attributed to metabolite C (three doublets, one double-doublet, one multiplet) suggests that the compound is another isopentane derivative. The chemical shifts observed for the double-doublet (119.6 ppm) and the multiplet (139.4 ppm) show that a carbon-carbon double bond connects C-2 (coupled to two ${}^{\text{13}}\text{C}$ neighbours) and C-3 (coupled to three ${}^{\text{13}}\text{C}$ neighbours) of the molecule.

The ${}^{\text{1}}\text{H}$ NMR chemical shifts of metabolite C are revealed by HMQC and HMQC-TOCSY experiments showing two singlets at 1.75 and 1.71 ppm, and a spin system comprising signals at 5.43 and 4.45 ppm. In conjunction with the chemical shifts, this correlation pattern shows that metabolite C is a dimethylallyl derivative.

The ${}^{\text{13}}\text{C}$ and ${}^{\text{1}}\text{H}$ NMR chemical shifts of an authentic sample of dimethylallyl diphosphate (DMAPP) are identical to the chemical shifts of the signals attributed to metabolite C. This leaves no doubt that metabolite C is [$^{\text{U}}\text{-}{}^{\text{13}}\text{C}_5$]dimethylallyl diphosphate (DMAPP).

The NMR data of metabolite A (IPP) and metabolite C (DMAPP) are summarized in Tables 6 and 7.

Table 6. NMR data of isopentenyl diphosphate (IPP)

| Position | Chemical shifts, ppm | | | Coupling constants, Hz | | | | |
|----------|-----------------------------|------------------------------|------------------------------|------------------------|-----------------|-----------------|-----------------|------------------------------|
| | ¹ H ^a | ¹³ C ^b | ³¹ P ^c | J _{PC} | J _{HH} | J _{PP} | J _{PH} | J _{CC} ^d |
| 1 | 4.00 | 64.1 | | 4.9 | 6.6 | | 6.6 | 34 |
| 2 | 2.31 | 37.8 | | 8.0 | 6.7 | | | 40, 40 |
| 3 | | 143.3 | | | | | | 71, 41, 41 |
| 4 | 4.73 | 111.6 | | | | | | 71 |
| 5 | 1.68 | 21.6 | | | | | | 41 |
| P | | | - 7.8 | | | nd | | |
| P | | | - 11.9 | | | 19,5 | | |

^areferenced to external trimethylsilylpropane sulfonate.

^breferenced to external trimethylsilylpropane sulfonate.

^creferenced to external 85 % orthophosphoric acid.

^dobserved with [U-¹³C₅]IPP

Table 7. NMR data of dimethylallyl diphosphate (DMAPP)

| Position | Chemical shifts, ppm | | | Coupling constants, Hz | | | | |
|----------|-----------------------------|------------------------------|------------------------------|------------------------|-----------------|-----------------|-----------------|------------------------------|
| | ¹ H ^a | ¹³ C ^b | ³¹ P ^c | J _{PC} | J _{HH} | J _{PP} | J _{PH} | J _{CC} ^d |
| 1 | 4.45 | 62.7 | | 3.6 | 6.6 | | 6.6 | 47 |
| 2 | 5.43 | 119.6 | | 9.0 | 7.2 | | | 75, 48 |
| 3 | | 139.4 | | | | | | nd |
| 4 | 1.75 | 24.9 | | | | | | 42 |
| 5 | 1.71 | 17.1 | | | | | | 41 |
| P | | | - 9.1 | | | 21.7 | | |
| P | | | - 6.4 | | | 21.5 | | |

^areferenced to external trimethylsilylpropane sulfonate.

^breferenced to external trimethylsilylpropane sulfonate.

^creferenced to external 85 % orthophosphoric acid.

^dobserved with [U-¹³C₅]DMAPP

Example 31

Cloning of the *ispG* gene (fragment) from *Arabidopsis thaliana*

RNA is isolated from 1 g of 2 weeks old *Arabidopsis thaliana* var. Columbia plants (stems and leafs) by published procedures (Logemann et al. 1987).

A mixture containing 2.75 µg RNA, 50 nmol dNTP's, 1 µg random hexameric primer, 1 µg T₁₅-primer and 20 % first strand 5x buffer (Promega) in a total volume of 50 µl is incubated for 5 min. at 95 °C, cooled on ice and 500 U M-MLV reverse transcriptase (Promega) are added. The mixture is incubated for 1 h at 42 °C. After incubation at 92 °C for 5 min, RNase A (20 U) and RNase H (2 U) are added and the mixture is incubated for 30 min. at 37 °C.

The resulting cDNA (1 µl of this mixture) is used for the amplification of *ispG* by PCR.

The *A. thaliana* ORF *ispG* (accession no. dbj AB005246) without the coding region for the putative leader sequence from basepair (bp) position 2889 to 6476 is amplified by PCR using cDNA from *A. thaliana* as template. The reaction mixture contains 25 pmol of primer CCTGCATCCGAAGGAAGCCC, 25 pmol of primer CAGTTTCAAAGAACGGCCC, 1 µl of cDNA, 2 U of Taq DNA polymerase (Eurogentec, Seraing, Belgium) and 20 nmol of dNTPs in a total volume of 100 µl in 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 95 °C. Then 40 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 20 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis. The PCR amplicate is purified with the PCR purification kit from Qiagen. 1.7 µg of purified PCR product are obtained.

The PCR amplicate is used as template for a second PCR reaction. The reaction mixture contains 25 pmol of primer TGAATCAGGATCCAAGACGGTGAGAAGG, 25 pmol of primer TCCGTTGGTACCCACTCATCAGCCACGG, 2 µl of the first PCR amplification, 2 U of Taq DNA polymerase (Eurogentec, Seraing, Belgium) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 95 °C. Then 40 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C follow. After further incubation for 20 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with PCR purification kit from Qiagen. 1.4 µg of purified PCR product are obtained. 2.0 µg of the vector pQE30 and 1.4 µg of the purified PCR product are digested with *Bam*H*I* and *Kpn*I in order to produce cohesive ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of vector DNA and 12 ng of PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pQEgcpEara. The ligation mixture is incubated over night at 4 °C. 2 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue and M15[pREP4] (Zamenhof *et. al.*, 1972) cells. The plasmid pQEgcpEara is isolated as described above. 7 µg of plasmid DNA are obtained.

The DNA insert of the plasmid pQEgcpEara is sequenced as described above. The DNA sequence is found not to be identical with the sequence in the database (accession no. dbj AB005246, see Annex L).

Example 32

Screening of IspG (GcpE) enzyme activity

0.2 g cells of XL1-pACYClytBgcpE are suspended in 1 ml 50 mM Tris hydrochloride, pH 7.4 and 2 mM DTT, cooled on ice and sonified 3 × 7 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 80 % duty cycle output, control value of 4 output. The suspension is centrifuged at 14000 rpm for 15 minutes. The supernatant is used as crude cell extract in assays described as follows.

The assay mixture contains 100 mM Tris hydrochloride, pH 7.4, 1.2 mM dithiothreitol, 10 mM NaF, 1 mM CoCl₂, 2 mM NADH, 20 mM (18 µCi mol⁻¹) [2-¹⁴C]2C-methyl-erythritol 2,4-cyclodiphosphate, 0.5 mM pamidronate and 100 µl crude cell extract of XL1-pACYClytBgcpE in a total volume of 150 µl. The mixture is incubated for 10 to 45 min at 37 °C and cooled on ice. 10 µl of 30 % (g/v) trichloroacetic acid are added and the mixture is neutralized with 20 µl of 1 M NaOH. The mixture is centrifuged at 14.000 rpm for 10 minutes. Aliquots of 130 µl of the supernatant are analyzed by reversed-phase ion-pair HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 × 250 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany).

The column is developed with a linear gradient of 7-21 % (v/v) methanol in 20 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0 at a flow rate of 1 ml min⁻¹ and further with a linear gradient of 21-49 % (v/v) methanol in 15 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0. After washing the column with 49 % (v/v) methanol in 5 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0, the column is equilibrated with 7 % (v/v) methanol in 20 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0. The effluent is monitored by a continuous-flow radio detector (Beta-RAM, Biostep GmbH, Jahnsdorf, Germany). The retention volumes of 2C-methyl-erythritol 2,4-cyclodiphosphate, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate, DMAPP/ IPP are 18, 24 and 39 ml respectively.

After 10 minutes of incubation, about 13 % of 2C-methyl-erythritol 2,4-cyclodiphosphate have been converted into 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (5 %) and into DMAPP/IPP (8 %), respectively.

After 45 min, no 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate, but about 21 % of DMAPP/IPP was found in the assay mixture.

Example 33

Screening of IspH (LytB) activity

Assay mixtures contain 100 mM Tris hydrochloride, pH 7.4, 1.2 mM DTT, 10 mM NaF, 0.5 mM NADH, 60 µM FAD, 0.004 µM (18 µCi µmol⁻¹) [2-¹⁴C]1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate, 0.5 mM pamidronate (Dunford *et al.*, 2001) and 20 µl of crude cell extract of M15-pMAL⁺lytB cells (prepared as described in example 2) in a total volume of 150 µl. The mixture is incubated for 30 min at 37 °C. The reaction is terminated by cooling on ice, addition of 10 µl of 30 % (g/v) trichloroacetic acid and immediate neutralization with 20 µl 1 M sodium hydroxide. The mixtures are centrifuged and aliquots (130 µl) of the supernatant are analyzed by reversed-phase ion-pair HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) analyzed by reversed-phase ion-pair HPLC using a column of Multospher 120 RP 18-AQ-5 (4.6 x 250 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany). The column is developed with a linear gradient of 7-21 % (v/v) methanol in 20 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0 at a flow rate of 1 ml min⁻¹ and further with a linear gradient of 21-49 % (v/v) methanol in 15 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0. After

washing the column with 49 % (v/v) methanol in 5 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0, the column is equilibrated with 7 % (v/v) methanol in 20 ml of 10 mM tetra-n-butylammonium hydrogen phosphate, pH 6.0. The effluent is monitored by a continuous-flow radio detector (Beta-RAM, Biostep GmbH, Jahnsdorf, Germany).

Under standard assay conditions, the HPLC peak corresponding to the substrate 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate is completely diminished, whereas two new peaks corresponding to DMAPP and IPP appear, when crude cell extract of *E. coli* M15-pMAL_{lytB} cells is used as protein source. No conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into DMAPP and IPP can be observed, when crude cell extract of *E. coli* wild-type is used as protein source. This findings clearly show that the FAD and NADH- or NADPH-dependent conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into DMAPP and IPP is catalyzed by the recombinant LytB protein. The addition of pamidronate in the assay mixtures prevents a further metabolization of IPP and DMAPP by highly active prenyl transferases present in crude *E. coli* extracts and affects therefore the complete conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate into DMAPP and IPP.

Example 34

Construction of a vector carrying the *dxs*, *xytB* and *ispC* genes capable for the transcription and expression thereof

The *B. subtilis* ORF *dxs* (accession no. dbj D84432) from base pair (bp) position 193991 to 195892 is amplified by PCR using pBSDXSBACSU plasmid DNA as template (see patent application PCT/EP00/07548). The reaction mixture contains 10 pmol of the primer 5'-GGCGACTCGCGAGAGGAGAAATTAAACCATGGATCTTTATCAATACAGGACC-3', 10 pmol of the primer 5'-GGCACCCGGCCGTATGATCCAATTCCCTTGTTG-3', 20 ng DNA of pBSDXSBACSU plasmid, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 120 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the

mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.4 µg of the vector pACYC184 (Chang and Cohen 1978, NEB) and 1.8 µg of the purified PCR product are digested with *Nru*I and *Eag*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 19 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYCdxs. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYCdxs is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYCdxs is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (dbj D84432).

2.0 µg of the vector pACYCdxs and 8 µg of the vector pBScyclo (see example XXX) are digested with *Eag*I and *Sal*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the digested and purified pACYCdxs vector DNA and 30 ng of a by DNA electrophoresis separated and purified 2.7 kb *Eag*I/*Sal*I fragment (containing the ORFs *xy*B and *isp*C from *E. coli*) are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYCdxsxyBispC. The ligation mixture is incubated for 2 h at 25 °C. 1 µl of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYCdxsxyBispC is isolated with the

plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYCdxsxyIBispC is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions.

Example 35

Construction of a vectors carrying the *dxs*, *xytB*, *ispC*, and *ispG* and optionally *ispH* genes capable for the transcription and expression thereof

The *E. coli* ORF *ispH* (*lytB*) (accession no. gb AE000113) from base pair (bp) position 5618 to 6568 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GCTTGCCTCGACGAGGAGAAATTAAACCATGCAGATCCTGTTGGCCACC-3', 10 pmol of the primer 5'-GCTGCTCTCGAGTTAACGACTTCACGAATATCG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 µl is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.5 µg of the vector pACYCdxsxyIBispC (see example 34) are linearized with *Sall* and 0.9 µg of the purified PCR product are digested with *Sall* and *Xhol* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

15 ng of the purified vector DNA and 18 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 µl of T4-Ligase buffer (Gibco) in a total volume of 10 µl, yielding the plasmid pACYCdxsxyIBispClytB. The ligation mixture is incubated for 2 h at 25 °C.

1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pACYCdxsxyIBispClytB is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pACYCdxsxyIBispClytB is sequenced by the automated dideoxynucleotide method using an ABI Prism 377TM DNA sequencer from Perkin Elmer with the ABI PrismTM Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000113).

The *E. coli* ORF *ispG* (*gcpE*) (accession no. gb AE000338) from base pair (bp) position 372 to 1204 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GGTCGAGTCGACGAGGAGAAATTACCATGCATAACCAGGCTCCAATTC-3', 10 pmol of the primer 5'-CCCATCCTCGAGTTATTTCAACCTGCTGAACGTC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

Each 2.0 μ g of the vectors pACYCdxsxyIBispC (see example 34) and pACYCdxsxyIBispClytB (see above) are linearized with *Sal*I and 1.1 μ g of the purified PCR product are digested with *Sal*I and *Xba*I in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

18 ng of the purified vector DNAs and 23 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmids pACYCdxsxyIBispCgcpE and pACYCdxsxyIBispClytBgcpcE. The ligation mixtures are incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into

electrocompetent *E. coli* XL1-Blue cells. The plasmids pACYCdxsxyIBispCgcpE and pACYCdxsxyIBispClytBgcpE are isolated with the plasmid isolation kit from Qiagen.

The DNA inserts of the plasmids pACYCdxsxyIBispCgcpE and pACYCdxsxyIBispClytBgcpE are sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. They are identical with the DNA sequence of the database entry (gb AE000338).

Example 36

Incorporation experiment with recombinant *Escherichia coli* XL1-pACYCdxsxyIBispCgcpE using [U-¹³C₆]glucose

0.2 litre of Terrific Broth (TB) medium containing 5 mg of chloramphenicol are inoculated with *E. coli* strain XL1-Blue harbouring the plasmid pACYCdxsxyIBispCgcpE. The cells are grown in a shaking culture at 37 °C overnight. At an optical density (600 nm) of 1.7-2.4 a solution containing 1 g of lithium lactate (10 mmol), 200 mg [U-¹³C₆]glucose (1.1 mmol) at a final volume of 24 ml (pH=7.4) are added continuously within 2 hours. Then, after 1 hour an aliquot of 40 ml was taken and centrifuged for 20 min at 5,000 rpm and 4 °C. The cells are washed with water containing 0.9 % NaCl and centrifuged as described above. The cells are suspended in 700 µl of a mixture of methanol-d₄ and D₂O (6:4, v/v) containing 10 mM NaF, cooled on ice and sonified 3 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 90 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15,000 rpm for 15 min. NMR spectra of the cell free extracts are recorded directly with a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). In order to avoid degradation during work-up, the structures of the products are determined by NMR spectroscopy without further purification.

The ¹³C-NMR spectra showed signals accounting for 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (cf. Tables 2 and 4, example 18) as major product. A formation of 2C-methyl-D-erythritol 2,4-cyldiphosphate could not be detected.

Example 37

Incorporation experiment with recombinant *Escherichia coli* XL1-pACYCdxsxyIBispClytBgcpE using glucose

Example 36 can be carried out with recombinant *Escherichia coli* XL1-pACYCdxsxyIBispClytBgcpE using glucose for converting glucose to isopentenyl diphosphate and/or dimethylallyl diphosphate.

Example 38

Cloning of the *ispG* gene of *Escherichia coli* and expression as maltose binding fusion protein (MBP-IspG)

The *E. coli* ORF *ispG* (*gcpE*) (accession no. gb AE000338) from base pair (bp) position 372 to 1204 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-GAACCGGAATTCATGCATAACCAGGCTCCAATT-3', 10 pmol of the primer 5'-CGAGGCAGATCCATCACG-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 60 sec at 94 °C, 60 sec at 50 °C and 90 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.2 μ g of the vector pMAL-C2 (NEB) and 0.8 μ g of the purified PCR product are digested with *Eco*RI and *Bam*HI in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR purification kit from Qiagen.

20 ng of the purified vector DNA and 15 ng of the purified PCR product are ligated together

with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pMALgcpE. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pMALgcpE is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pMALgcpE is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000338).

Example 39

Cloning of the *ispH* gene of *Escherichia coli* and expression as maltose binding fusion protein (MBP-*IspH*)

The *E. coli* ORF *ispH* (*lytB*) (accession no. gb AE000113) from base pair (bp) position 5618 to 6568 is amplified by PCR using chromosomal *E. coli* DNA as template. The reaction mixture contains 10 pmol of the primer 5'-TGGAGGGATCCATGCAGATCCTGTTGCCACC-3', 10 pmol of the primer 5'-GCATTCTGCAGAACTTAGGC-3', 20 ng of chromosomal DNA, 2 U of Taq DNA polymerase (Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μ l containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-hydrochloride, pH 8.8 and 0.1 % (w/w) Triton X-100.

The mixture is denatured for 3 min at 94 °C. Then 30 PCR cycles for 45 sec at 94 °C, 45 sec at 50 °C and 60 sec at 72 °C followed. After further incubation for 10 min at 72 °C, the mixture is cooled to 4 °C. An aliquot of 2 μ l is subjected to agarose gel electrophoresis.

The PCR amplicate is purified with the PCR purification kit from Qiagen (Hilden).

2.2 μ g of the vector pMAL-C2 (NEB) and 0.7 μ g of the purified PCR product are digested with *Bam*H*I* and *Pst**I* in order to produce DNA fragments with overlapping ends. The restriction mixtures are prepared according to the conditions supplied by the customer (NEB) and are incubated for 3 h at 37 °C. Digested vector DNA and PCR product are purified using the PCR

purification kit from Qiagen.

20 ng of the purified vector DNA and 14 ng of the purified PCR product are ligated together with 1 U of T4-Ligase (Gibco), 2 μ l of T4-Ligase buffer (Gibco) in a total volume of 10 μ l, yielding the plasmid pMALlytB. The ligation mixture is incubated for 2 h at 25 °C. 1 μ l of the ligation mixture is transformed into electrocompetent *E. coli* XL1-Blue cells. The plasmid pMALlytB is isolated with the plasmid isolation kit from Qiagen.

The DNA insert of the plasmid pMALlytB is sequenced by the automated dideoxynucleotide method using an ABI Prism 377™ DNA sequencer from Perkin Elmer with the ABI Prism™ Sequencing Analysis Software from Applied Biosystems Divisions. It is identical with the DNA sequence of the database entry (gb AE000113).

Example 40

Preparation and purification of recombinant IspG maltose binding fusion protein (MRP-IspG)

0.5 liter of Luria Bertani (LB) medium containing 90 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harboring plasmid pMALgcpE. The culture is grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.7, the culture is induced with 2 mM IPTG. The culture is grown for further 5 h. The cells are harvested by centrifugation for 20 min at 5,000 rpm and 4 °C. The cells are washed with 20 mM Tris hydrochloride pH 7.4, centrifuged as above and frozen at -20 °C for storage.

2 g of the cells are thawed in 20 ml of 20 mM Tris hydrochloride pH 7.4, 0.2 M sodium chloride and 0.02 % (g/v) sodium acid (buffer A) in the presence of 1 mg ml^{-1} lysozyme and 100 $\mu\text{g ml}^{-1}$ DNaseI. The mixture is incubated at 37 °C for 30 min, cooled on ice and sonified 6 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 70 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15, 000 rpm at 4 °C for 30 min. The cell free extract is applied on a column of amylose resin FF (column volume 25 ml, NEB) previously equilibrated with buffer A at a flowrate of 2 ml min^{-1} . The column is washed with 130 ml of buffer A. MRP-IspG is eluted with a linear gradient of 0-10 mM maltose in buffer A. MRP-IspG containing fractions are combined according to SDS-PAGE and dialyzed overnight against 100 mM Tris hydrochloride pH 7.4. The homogeneity of MRP-IspG is judged by SDS-

PAGE. One band at 84 kDa is visible, which is in line with the calculated molecular mass. The yield of pure MRP-IspG is 9 mg.

Example 41

Preparation and purification of recombinant IspH maltose binding fusion protein (MRP-IspH)

0.5 liter of Luria Bertani (LB) medium containing 90 mg of ampicillin are inoculated with 10 ml of an overnight culture of *E. coli* strain XL1-Blue harboring plasmid pMALIytB. The culture is grown in a shaking culture at 37 °C. At an optical density (600 nm) of 0.7, the culture is induced with 2 mM IPTG. The culture is grown for further 5 h. The cells are harvested by centrifugation for 20 min at 5,000 rpm and 4 °C. The cells are washed with 20 mM Tris hydrochloride pH 7.4, centrifuged as above and frozen at -20 °C for storage.

2 g of the cells are thawed in 20 ml of 20 mM Tris hydrochloride pH 7.4, 0.2 M sodium chloride and 0.02 % (g/v) sodium acid (buffer A) in the presence of 1 mg ml⁻¹ lysozyme and 100 µg ml⁻¹ DNaseI. The mixture is incubated at 37 °C for 30 min, cooled on ice and sonified 6 x 10 sec with a Branson Sonifier 250 (Branson SONIC Power Company) set to 70 % duty cycle output, control value of 4 output. The suspension is centrifuged at 15, 000 rpm at 4 °C for 30 min. The cell free extract is applied on a column of amylose resin FF (column volume 25 ml, NEB) previously equilibrated with buffer A at a flowrate of 2 ml min⁻¹. The column is washed with 130 ml of buffer A. MRP-IspH is eluted with a linear gradient of 0-10 mM maltose in buffer A. MRP-IspH containing fractions are combined according to SDS-PAGE and dialyzed overnight against 100 mM Tris hydrochloride pH 7.4. The homogeneity of MRP-IspH is judged by SDS-PAGE. One band at 78 kDa is visible, which is in line with the calculated molecular mass. The yield of pure MRP-IspH is 14 mg.

Example 42

Synthesis of 1-hydroxy-2-methyl-but-2-enyl-4-diphosphate (see Fig. 7)

4-Chloro-2-methyl-2-buten-1-al (Choi et al. (1999) J. Org. Chem. 64, 8051-8053)

A solution containing 1.17 ml of 2-methyl-2-vinyl-oxirane (12 mmol), 1.6 g of CuCl₂ (12 mmol) and 510 mg of LiCl (12 mmol) in 10 ml of ethylacetate was heated to 80 °C for 30 min. The

reaction was stopped by adding 50 g of ice. The mixture was filtered through a sintered glass funnel under reduced pressure. 100 ml of CH_2Cl_2 was added and the organic phase was separated. The aqueous layer was extracted two times with 100 ml of CH_2Cl_2 . The combined organic phase was dried over anhydrous MgSO_4 , filtered, and concentrated. The crude product was purified by chromatography over silica gel (CH_2Cl_2 , 3 x 37 cm) to yield 0.755 g of a yellow liquid (6.4 mmol, 53 %).

^1H NMR (CDCl_3 , 500 MHz) δ 9.43 (s, 1H), 6.50 (t, $J=7.5$ Hz, 1H), 4.24 (d, $J=7.5$ Hz, 2H),

1.77 (s, 3H)

^{13}C NMR (CDCl_3 , 125 MHz) δ 194.3, 145.7, 141.1, 38.6, 9.1

4-Chloro-2-methyl-2-buten-1-al-dimethyl-acetal

A solution of 184 mg 4-chloro-2-methyl-2-buten-1-al (1.55 mmol), 600 μl of HC(OMe)_3 (5.6 mmol) and a catalytic amount of p-TsOH was incubated for 3 h at roomtemperature. The crude mixture was purified by chromatography over silica gel (n-hexane/ethylacetate 7:3) to yield 177 mg of a colourless liquid (1.08 mmol, 72 %).

^1H NMR (CDCl_3 , 500 MHz) δ 5.78 (t, 1H, $J=7.9$), 4.47 (s, 1H), 4.15 (d, $J=7.9$ Hz, 2H), 3.33

(s, 6 H), 1.73 (s, 3H)

^{13}C NMR (CDCl_3 125 MHz) δ 137.6, 124.4, 106.0, 53.5, 39.6, 11.4

(E)-3-Formyl-2-buten-1-diphosphate triammonium salt (Davisson et al. (1986) *J. Org. Chem.*, 51, 4768)

To a solution of 4-chloro-2-methyl-2-buten-1-al-dimethyl-acetal chloride (25 mg, 0.15 mmol) in 250 μl of MeCN a solution of 0.162 g (0.18 mmol) of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate in 400 μl of MeCN was added slowly at room temperature, leading to an orange-red solution. After 2 h the reaction was finished and the solvent was removed under reduced pressure. The orange oil was dissolved in 3 mL of H_2O and passed through a column of DOWEX 50 WX8 (1 x 4 cm) cation-exchange resin (NH_4^+ form) that has been equilibrated with 20 mL of 25 mM NH_4HCO_3 . The column was eluted with 20 mL of 25 mM NH_4HCO_3 . The resulting solution was lyophilized. The obtained solid was dissolved in 2 ml water and acidified with aqueous HCl to pH=3. After 2 minutes the solution was neutralized and lyophilized.

^1H NMR (D_2O , 360 MHz) δ 9.37 (s, 1H), 6.86 (t, 1H, 5.6Hz), 4.85 (dd, $J=7.9$, $J=5.8$ Hz, 2H),

1.72 (s, 3H)

^{13}C NMR (D_2O , 90 MHz) δ 199.2, 153.1 (d, $J=7.5$ Hz), 138.5, 63.2 (d, $J=4.9$), 8.5

[1-³H]1-hydroxy-2-methyl-but-2-enyl-4-diphosphate

A solution containing 50 mCi (15 µmol) NaBH₃T, 15 µmol 3-formyl-2-buten-1-diphosphate triammonium salt and 100 mM Tris/HCl pH=8 was incubated for 30 minutes at room temperature. The solution was acidified by adding 1 M HCl to pH=2. After 2 minutes the solution was neutralized by adding 1 M NaOH.

The product was characterized by ion-exchange chromatography (see examples 20 and 25).

Example 43**γδ T cell stimulation assays**

PBMCs from healthy donors (donor A and donor B) are isolated from heparinized peripheral blood by density centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Freiburg, Germany). 5 × 10⁵ PBMCs/well are cultivated in 1 mL RPMI 1640 medium supplemented with 10% human AB serum (Klinik rechts der Isar, München, Germany), 2 mM L-glutamine, 10 µM mercaptoethanol. Amounts of recombinant human IL-2 (kindly provided by Eurocetus, Amsterdam, The Netherlands) and substrates are varied from 1 to 10 U and 10 to 0.1 µM, respectively. 20 µM IPP (Echelon, Research Laboratories Inc., Salt Lake City, USA) serves as a positive control whereas medium alone serves as negative control. Incubation is done for seven days at 37 °C in the presence of 7% CO₂. The harvested cells are double-stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody Vδ2 TCR and phycoerythrin (PE)-conjugated monoclonal CD3 antibody. The cells are analyzed using a FACScan supported with Cellquest (Becton Dickinson, Heidelberg, Germany).

The substrates (E)-1-hydroxy-3-methyl-but-2-enyl 4-diphosphate (HMBPP) and 3-formyl-but-2-enyl 1-diphosphate (Aldehyd) were prepared synthetically as described above.

It is found that both synthetically prepared substrates (HMBPP and Aldehyd) show at least double stimulation compared to IPP when used at a concentration that is 200-fold lower than the concentration of the IPP sample (Table 8).

Table 8. Activation of $\gamma\delta$ T-cells by phosphororganic compounds

| Substrate | Concentration [μ M] | IL-2 [U] | % $\gamma\delta$ T-cells | |
|-------------|-----------------------------|-------------|--------------------------|---------|
| | | | Donor A | Donor B |
| Medium | - | 1 | 1.51 | 2.75 |
| Medium | - | 5 | 1.45 | 2.23 |
| Medium | - | 10 | 1.32 | 1.68 |
| IPP | 20 | 1 | 8.19 | 6.24 |
| IPP | 20 | 5 | 14.42 | 9.32 |
| IPP | 20 | 10 | 16.6 | 11.86 |
| IPP | 1 | 1 | 1.56 | 2.22 |
| IPP | 1 | 5 | 1.59 | 2.67 |
| IPP | 1 | 10 | 1.71 | 2.19 |
| IPP | 0.1 | 1 | 1.3 | 2.15 |
| IPP | 0.1 | 5 | 1.3 | 2.26 |
| IPP | 0.1 | 10 | 1.01 | 2.54 |
| HMBPP | 10 | 1 | 3.3 | 31.42 |
| HMBPP | 10 | 5 | 17.38 | 63.48 |
| HMBPP | 10 | 10 | 24.94 | 63.34 |
| HMBPP | 1 | 1 | 5.57 | 35.34 |
| HMBPP | 1 | 5 | 14.4 | 54.12 |
| HMBPP | 1 | 10 | 19.85 | 55.90 |
| HMBPP | 0.1 | 1 | 11.78 | 32.21 |
| HMBPP | 0.1 | 5 | 22.92 | 44.69 |
| HMBPP | 0.1 | 10 | 34.69 | 36.33 |
| HMBPP/IPP | 0.5/0.5 | 1 | 7 | 30.35 |
| HMBPP/IPP | 0.5/0.5 | 5 | 15.38 | 53.76 |
| HMBPP/IPP | 0.5/0.5 | 10 | 24.19 | 46.58 |
| Aldehyd | 10 | 1 | 12.19 | 30.69 |
| Aldehyd | 10 | 5 | 34.69 | 30.33 |
| Aldehyd | 10 | 10 | 38.99 | 38.85 |
| Aldehyd | 1 | 1 | 15.91 | 21.18 |
| Aldehyd | 1 | 5 | 40.13 | 30.76 |
| Aldehyd | 1 | 10 | 48.28 | 36.69 |
| Aldehyd | 0.1 | 1 | 10 | 13.54 |
| Aldehyd | 0.1 | 5 | 19.77 | 18.45 |
| Aldehyd | 0.1 | 10 | 21.93 | 25.82 |
| Aldehyd/IPP | 0.5/0.5 | 1 | 13.98 | 22.11 |
| Aldehyd/IPP | 0.5/0.5 | 5 | 33.94 | 32.06 |
| Aldehyd/IPP | 0.5/0.5 | 10 | 42.84 | 36.25 |

IPP: isopentenyl diphosphate

HMBPP: (E)-1-hydroxy-3-methyl-but-2-enyl 4-diphosphate

Aldehyd: (E)-3-formyl-but-2-enyl 1-diphosphate (prepared according to example 42)

Example 44

High through-put screening assay of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate

synthase (IspG) activity

Assay mixtures contain 20 mM potassium phosphate, pH 7.0, 0.4 mM NADH, 0.5 mM CoCl₂, 0.2 mM 2C-methyl-D-erythritol 2,4-cyclodiphosphate, and 50 µl protein in a total volume of 1 ml. The mixtures are incubated at 37 °C. The oxidation of NADH is monitored photometrically at 340 nm. Alternatively, the concentration of NADH is determined by measuring the relative fluorescence of NADH at 340 nm excitation/460 nm emission.

Example 45

High through-put screening assay of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (IspH) activity

Assay mixtures contain 20 mM potassium phosphate, pH 7.0, pH 8.0, 0.4 mM NADH, 20 µM FAD, 0.5 mM CoCl₂, 0.2 mM 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, and 50 µl protein in a total volume of 1 ml. The mixtures are incubated at 37 °C. The oxidation of NADH is monitored photometrically at 340 nm. Alternatively, the concentration of NADH is determined by measuring the relative fluorescence of NADH at 340 nm excitation/460 nm emission.

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Claims

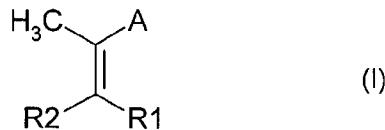
1. A protein in a form that is functional for the enzymatic conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-but enyl 4-diphosphate notably in its (*E*)-form.
2. The protein according to claim 1, wherein it is functional for said conversion in the presence of NADH and/or NADPH.
3. The protein according to claim 2, wherein it is functional for said conversion in the presence of Co^{2+} .
4. The protein according to one of claims 1 to 3, wherein it has a sequence encoded by the *ispG* (formerly *gcpE*) gene of *E. coli* or a function-conservative homologue of said sequence.
5. A protein in a form that is functional for the enzymatic conversion of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably in its (*E*)-form, to isopentenyl diphosphate and/or dimethylallyl diphosphate.
6. The protein according to claim 5, wherein it is in a form functional for said conversion in the presence of FAD and NAD(P)H.
7. The protein according to claim 6, wherein it is in a form functional for said conversion in the presence of a metal ion selected from the group of manganese, iron, cobalt, or nickel ion.
8. The protein according to one of claims 5 to 7, wherein it has a sequence encoded by the *ispH* (formerly *lytB*) gene of *E. coli* or a function-conservative homologue of said sequence.
9. The protein according to one of claims 1 to 8, wherein it is a plant protein, notably from *Arabidopsis thaliana*.

10. The protein according to one of claims 1 to 8, wherein it is a bacterial protein, notably from *E. coli*.
11. The protein according to one of claims 1 to 8, wherein it is a protozoal protein, notably from *Plasmodium falciparum*.
12. Purified isolated nucleic acid encoding the protein according to one of claims 1 to 4 and/or the protein according to one of claim 5 to 8 with or without introns.
13. A DNA expression vector containing the sequence of the nucleic acid according to claim 12.
14. Use of a protein according to one of claims 1 to 11 for screening a chemical library for an inhibitor of the biosynthesis of isoprenoids.
15. Cells, cell cultures, organisms or parts thereof recombinantly endowed with the sequence of the nucleic acid according to claim 12 or with the vector according to claim 13, wherein said cell is selected from the group consisting of bacterial, protozoal, fungal, plant, insect and mammalian cells.
16. Cells, cell cultures, organisms or parts thereof according to claim 15, wherein it is recombinantly endowed with a vector containing a nucleic acid sequence encoding a protein according to one of claims 1 to 4 and/or a protein according to one of claims 5 to 8, and wherein said cell is optionally further endowed with at least one gene selected from the following group: *dxs*, *dxr*, *ispD* (formerly *ygbP*); *ispE* (formerly *ychB*); *ispF* (formerly *ygbB*) of *E. coli* or a function-conservative homologue thereof, or a function-conservative fusion, deletion or insertion variant of any of the above genes.
17. Cells, cell cultures, or organisms or parts thereof transformed or transfected for an increased rate of formation of 1-hydroxy-2-methyl-2-butanyl 4-diphosphate, notably in its (*E*)-form, compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection.

18. Cells, cell cultures, or organisms or parts thereof transformed or transfected for an increased rate of conversion of (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate to isopentenyl diphosphate and/or dimethylallyl diphosphate compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection.
19. Cells, cell cultures, or organisms or parts thereof according to claim 15 transformed or transfected for an increased expression level of the protein of one of claims 1 to 4 and/or the protein of one of claims 5 to 8 compared to cells, cell cultures, or organisms or parts thereof absent said transformation or transfection.
20. Cells, cell cultures or organisms or parts thereof in accordance with claim 15 or 16, characterized by the recombinant endowment with sets of genes as follows:
ispC (formerly *dxr*), *ispD*, *ispE*, *ispF*, *ispG* (formerly *gcpE*); or
ispC, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* (formerly *lytB*); or
dxs, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*; or
dxs, *ispC*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH*; or
dxs, *ispC*, *ispG*, or
dxs, *ispC*, *ispG*, *ispH*
of *E. coli* or a function-conservative homologue thereof and/or a function-conservative fusion, deletion or insertion variant of any of the above genes.
21. Cells, cell cultures or organisms or parts thereof in accordance with claim 20, characterized by further recombinant endowment(s) with gene(s) being functional for biosynthetic steps downstream from the C5 isoprenoids.
22. Cells, cell cultures or organisms or parts thereof in accordance with one of claims 15 to 21, wherein at least one gene of said recombinant endowments is equipped with artificial ribosomal binding site(s) for expression of the corresponding gene product(s) at a rate enhanced compared to the rate in the absence of the artificial ribosomal binding site(s).
23. Cells, cell cultures or organisms or parts thereof in accordance with one of claims 15 to 22, wherein at least one of said recombinant endowments is due to a high copy replication vector.

24. Cells, cell cultures or organisms or parts thereof in accordance with one of claims 15 to 23, wherein they are of bacterial, protozoal, fungal, plant or animal origin.
25. Use of the cells, cell cultures or organisms, or parts thereof in accordance with one of claims 15 to 24 or disruption products thereof for the enhanced rate of *in vivo* formation or for the efficient *in vitro* production of an, optionally isotopically labelled, biosynthetic intermediate or product of the non-mevalonate isoprenoid biosynthetic pathway, optionally by feeding 1-deoxy-D-xylulose or glucose that may be isotopically labelled.
26. Use according to claim 25, wherein said intermediate or product is a C5-isoprenoid intermediate compound; or a >C5-isoprenoid compound; or a terpenoid compound.
27. Use according to one of claims 25 or 26, wherein the rate of formation or production is enhanced by providing a source for CTP.
28. Use according to claim 27, wherein the source for CTP is cytidine and/or uridine and/or cytosine and/or uracil and/or ribose and and/or ribose 5-phosphate and/or any biosynthetic precursor of CTP.
29. Use according to one of claims 25 to 28, wherein the rate of formation or production is enhanced by providing a source for phosphorylation enhancement.
30. Use according to claim 29, wherein the source for phosphorylation enhancement is glycerol 3-phosphate and/or phosphoenolpyruvate and/or inorganic phosphate and/or inorganic pyrophosphate and/or any organic phosphate or pyrophosphate.
31. Use according to one of claims 25 to 30, wherein the rate of formation or production is enhanced by providing a source for reduction equivalents.
32. Use according to claim 31, wherein the source for reduction equivalents is succinate and/or lipids and/or glucose and/or glycerol and/or lactate.

33. Optionally isotope-labelled compound of the following formula I or a salt thereof:



whereby R¹ and R² are different from each other and one of R¹ and R² is hydrogen and the other is selected from the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂, -CH₂-O-PO(OH)₂, and -CH₂OH, and whereby A stands for -CH₂OH or -CHO.

34. The optionally isotope-labelled compound according to claim 33, wherein A stands for -CH₂OH.

35. The optionally isotope-labelled compound according to claim 33, wherein A stands for -CHO.

36. The optionally isotope-labelled compound according to one of claims 33 to 35, wherein R¹ is H and R² is selected from the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂ and -CH₂-O-PO(OH)₂.

37. The optionally isotope-labelled compound according to one of claims 33 to 35, wherein R² is H and R¹ is selected from the group consisting of -CH₂-O-PO(OH)-O-PO(OH)₂ and -CH₂-O-PO(OH)₂.

38. The optionally isotope-labelled compound according to one of claims 33 to 37, whereby said group consists of -CH₂-O-PO(OH)-O-PO(OH)₂.

39. Optionally isotope-labelled 1-hydroxy-2-methyl-2-butenyl 4-diphosphate salt or a protonated form thereof.

40. Optionally isotope-labelled (*E*)-1-hydroxy-2-methyl-2-butenyl 4-diphosphate salt or a protonated form thereof.

41. Optionally isotope-labelled (Z)-1-hydroxy-2-methyl-2-butenyl 4-diphosphate salt or a protonated form thereof.
42. Use of a compound according to one of claims 33 to 41, notably according to claim 38, for screening for genes, enzymes or inhibitors of the biosynthesis of isoprenoids or terpenoids, either *in vitro* in the presence of an electron donor or *in vivo*.
43. Use of a compound according to one of claims 33 to 41, notably according to claim 40, as an immunomodulatory agent.
44. Use of a compound according to one of claims 33 to 41, notably according to claim 40, for activating $\gamma\delta$ T cells.
45. Use of a compound according to one of claims 33 to 41, notably according to claim 40, for the preparation of a medicament.
46. Pharmaceutical composition containing a compound according to one of claims 33 to 41, notably according to claim 36 or 40, and a pharmaceutically acceptable carrier.
47. The pharmaceutical composition according to claim 46 further comprising an antibiotically active compound.
48. The pharmaceutical composition according to claim 47, wherein the antibiotically active compound is bacteriostatic.
49. The pharmaceutical composition according to claim 47, wherein the antibiotically active compound inhibits bacterial protein synthesis.
50. A method of treating a pathogen infection comprising administering a pharmaceutical composition according to one of claims 46 to 49.
51. Monoclonal or polyclonal antibody against a compound of one of claims 33 to 41.

52. A method of detecting a pathogen, notably in a body fluid, by using the antibody of claim 51.
53. Use of the cells, cell cultures or organisms or parts thereof in accordance with claims 15 to 24 for the production of a protein in an enzymatically competent form for the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate.
54. Use of the cells, cell cultures or organisms or parts thereof in accordance with claims 15 to 24 for the production of a protein in an enzymatically competent form for the conversion of (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate into isopentenyl diphosphate and/or dimethylallyl diphosphate.
55. Use of the cells, cell cultures or organisms or parts thereof in accordance with claims 15 to 24 for the production of proteins in an enzymatically competent form for the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate into isopentenyl diphosphate and/or dimethylallyl diphosphate.
56. A method of altering the expression level of the gene product(s) of *ispG* and/or *ispH* in cells comprising
 - (a) transforming host cells with the *ispG* and/or *ispH* gene,
 - (b) growing the transformed host cells of step (a) under conditions that are suitable for the efficient expression of *ispG* and/or *ispH*, resulting in production of altered levels of the *ispG* and/or *ispH* gene product(s) in the transformed cells relative to expression levels of untransformed cells.
57. Method of identifying an inhibitor of an enzyme functional for the conversion of 2C-methyl-D-erythritol 2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably its *E*-form, of the non-mevalonate isoprenoid pathway by the following steps:
 - (a) incubating a mixture containing said enzyme with its, optionally isotope-labeled, substrate 2C-methyl-D-erythritol-2,4-cyclodiphosphate under conditions suitable for said conversion in the presence and in the absence of a potential inhibitor,

- (b) subsequently determining the concentration of 2C-methyl-D-erythritol 2,4-cyclodiphosphate and/or 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, and
- (c) comparing the concentration in the presence and in the absence of said potential inhibitor.

58. Method of identifying an inhibitor of an enzyme functional for the conversion of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate, notably its E-form, to isopentenyl diphosphate or dimethylallyl diphosphate of the non-mevalonate isoprenoid pathway by the following steps:

- (a) incubating a mixture containing said enzyme with its, optionally isotope-labeled, substrate 1-hydroxy-2-methyl-2-butenyl 4-diphosphate under conditions suitable for said conversion in the presence and in the absence of a potential inhibitor,
- (b) determining the concentration of 1-hydroxy-2-methyl-2-butenyl 4-diphosphate and/or isopentenyl diphosphate or dimethylallyl diphosphate, and
- (c) comparing the concentration in the presence and in the absence of said potential inhibitor.

59. The method according to claim 58, wherein step (a) is carried out in the presence of FAD.

60. The method of one of claims 57 to 59, which further comprises preparing cells recombinantly endowed with a gene coding for said enzyme, culturing said cells, preparing a crude extract of said cells, and using said crude extract in step (a).

61. The method according to one of claims 57 to 60, wherein said enzyme is a plant enzyme.

62. The method according to one of claims 57 to 60, wherein said enzyme is an enzyme of *Plasmodium falciparum*.

63. The method according to one of claims 57 to 60, wherein said enzyme is a bacterial enzyme.

64. The method according to one of claims 57 to 60, wherein the incubation of step (a) is carried out in the presence of a sulphydryl reductant e.g. dithiothreitol.
65. The method according to one of claims 57 to 64, wherein the incubation in step (a) is carried out in the presence of a phosphatase inhibitor.
66. The method according to claim 65, wherein the phosphatase inhibitor is an alkali fluoride.
67. The method according to one of claims 57 to 66, wherein the incubation of step (a) is carried out in the presence of NADH or NADPH.
68. The method according to one of claims 57 to 67, wherein the incubation in step (a) is carried out in the presence of an inhibitor of an enzyme acting downstream of isopentenyl diphosphate or dimethylallyl diphosphate.
69. The method according to one of claims 57 to 68, wherein the incubation of step (a) is carried out in the presence of a salt selected from the group of Co^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} salts.
70. The method according to one of claims 57 to 69, wherein step (b) is carried out by reversed phase ion-pair HPLC chromatography.
71. The method according to one of claims 57 to 69, wherein step (b) is carried out by determining the consumption of NADH or NADPH.
72. The method according to one of claims 57 to 71, which is carried out on many potential inhibitors simultaneously or consecutively in a high-throughput screening.
73. A process for the efficient *in vivo* synthesis of 1-hydroxy-2-methyl-2-butanyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-butanyl 4-diphosphate; or isopentenyl

diphosphate or dimethylallyl diphosphate; optionally isotope labelled, in salt form or in protonated form, by the following steps:

- (a) culturing cells, preferably bacterial cells, recombinantly endowed in accordance with one of claims 15 to 24 for said synthesis for a predetermined period of time at a predetermined temperature;
- (b) optionally adding glucose to a predetermined final concentration and further culturing for a predetermined period of time;
- (c) harvesting the cells;
- (d) preparing a crude extract from the harvested cells;
- (e) separating and purifying optionally isotope-labelled 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate; or isopentenyl diphosphate; or dimethylallyl diphosphate; in salt form or in protonated form, optionally by preparative chromatography.

74. A process for screening chemical libraries for the presence or absence of inhibition of the biosynthesis of isoprenoids, notably by blocking the biosynthesis of the intermediates 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate and/or isopentenyl diphosphate and/or dimethylallyl diphosphate, said screening comprising:

- (a) culturing cells, preferably bacterial cells, recombinantly endowed in accordance with claim 15 for a predetermined period of time at a predetermined temperature;
- (b) optionally adding glucose to a predetermined final concentration and further culturing for a predetermined period of time;
- (c) harvesting the cells;
- (d) preparing a crude extract from the harvested cells;

whereby steps (a) to (d) are carried out in the presence and in the absence of a prospective inhibitor;

- (e) detecting difference(s) in the level(s) of 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate and/or isopentenyl diphosphate and/or dimethylallyl diphosphate; between the presence and absence of the prospective inhibitor; and

(f) correlating said detected difference(s) with the presence or absence of an above-defined inhibition.

75. Cells, cell cultures or organisms or parts thereof for the efficient formation of a biosynthetic product or intermediate of the non-mevalonate pathway to isoprenoids or terpenoids, characterized by

- first recombinant endowment with a gene functional for the biosynthesis of 1-deoxy-D-xylulose 5-phosphate from 1-deoxy-D-xylulose;
- capability for the uptake of 1-deoxy-D-xylulose; and
- recombinant endowment(s) with gene(s) being functional for the conversion of 1-deoxy-D-xylulose 5-phosphate into desired downstream C5-intermediate(s) of said pathway.

76. Cells, cell cultures or organisms or parts thereof in accordance with claim 75, wherein said gene(s) of said second recombinant endowment(s) code(s) for enzyme(s) for the formation of at least one of the following C5-intermediates of the non-mevalonate isoprenoid pathway:

- 2C-methyl-D-erythritol 4-phosphate;
- 4-diphosphocytidyl-2C-methyl-D-erythritol;
- 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate;
- 2C-methyl-D-erythritol 2,4-cyclodiphosphate;
- 1-hydroxy-2-methyl-2-but enyl 4-diphosphate;
- isopentenyl diphosphate;
- dimethylallyl diphosphate.

77. Cells, cell cultures or organisms or parts thereof in accordance with claim 75 or 76, characterized by the recombinant endowment with sets of genes as follows:

- xylB*, *dxr*, or
- xylB*, *dxr*, *ispD* (formerly *ygbP*); or
- xylB*, *dxr*, *ispD*, *ispE* (formerly *ychB*), or
- xylB*, *dxr*, *ispD*, *ispE*, *ispF* (formerly *ygbB*); or
- xylB*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG* (formerly *gcpE*); or
- xylB*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* (formerly *lytB*)

of *E. coli* or a function-conservative homologue thereof and/or a function-conservative fusion, deletion or insertion variant of any of the above genes.

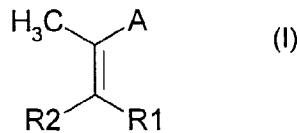
78. Cells, cell cultures or organisms or parts thereof in accordance with claim 75, characterized by the recombinant endowment with *xytB* and *ispG* (formerly *gcpE*) and optionally at least one gene selected from the following group: *dxr*; *ispD* (formerly *ygbP*); *ispE* (formerly *ychB*); *ispF* (formerly *ygbB*) of *E. coli* or a function-conservative homologue thereof, or a function-conservative fusion, deletion or insertion variant of any of the above genes.
79. Cells, cell cultures or organisms or parts thereof in accordance with claim 75, characterized by the recombinant endowment with *xytB* and *ispH* (formerly *lytB*) and optionally at least one gene selected from the following group: *dxr*; *ispD* (formerly *ygbP*); *ispE* (formerly *ychB*); *ispF* (formerly *ygbB*); *ispG* (formerly *gcpE*) of *E. coli* or a function-conservative homologue thereof, or a function-conservative fusion, deletion or insertion variant of any of the above genes.
80. Cells, cell cultures or organisms or parts thereof in accordance with claim 75, characterized by the recombinant endowment with *xyl*, *ispG*, (formerly *gcpE*) and *ispH* (formerly *lytB*) and optionally at least one gene selected from the following group: *dxr*; *ispD* (formerly *ygbP*); *ispE* (formerly *ychB*); *ispF* (formerly *ygbB*); of *E. coli* or a function-conservative homologue thereof, or a function-conservative fusion, deletion or insertion variant of any of the above genes.
81. A process for the efficient *in vivo* synthesis of 2C-methyl-D-erythritol 4-phosphate; or 4-diphosphocytidyl-2C-methyl-D-erythritol; or 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; or 2C-methyl-D-erythritol 2,4-cyclodiphosphate; or 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate; or isopentenyl diphosphate or dimethylallyl diphosphate; optionally isotope labelled, in salt form or in protonated form, by the following steps:
 - (a) culturing cells, preferably bacterial cells, recombinantly endowed in accordance with one of claims 75 to 80 for said synthesis for a predetermined period of time at a predetermined temperature;

- (b) adding 1-deoxy-D-xylulose to a predetermined final concentration and further culturing for a predetermined period of time;
- (c) harvesting the cells;
- (d) preparing a crude extract from the harvested cells;
- (e) separating and purifying optionally isotope-labelled 2C-methyl-D-erythritol 4-phosphate; or 4-diphosphocytidyl-2C-methyl-D-erythritol; or 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; or 2C-methyl-D-erythritol 2,4-cyclodiphosphate; or 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate; or isopentenyl diphosphate; or dimethylallyl diphosphate; in salt form or in protonated form, by preparative chromatography.

82. The process according to one of claims 73, 74 or 81, wherein step (a) is carried out in terrific broth medium.
83. The process according to one of claims 73, 74, 81 or 82, wherein a source for CTP, preferably cytidine or uridine, is added in step (a).
84. The process according to one of claims 73, 74 or 81 to 83, wherein a source of phosphorylation activity, preferably glycerol 3-phosphate and/or inorganic phosphate, is added in step (a).
85. The process according to one of claims 73, 74 or 81 to 84 wherein a source of reduction equivalents, preferably succinate and/or lipids and/or glucose and/or glycerol and/or lactate is added in step (a).
86. A process for screening chemical libraries for the presence or absence of inhibition of the biosynthesis of isoprenoids, notably by blocking the biosynthesis of the intermediates 2C-methyl-D-erythritol 4-phosphate and/or 4-diphosphocytidyl-2C-methyl-D-erythritol and/or 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate and/or 2C-methyl-D-erythritol 2,4-cyclodiphosphate and/or 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate and/or isopentenyl diphosphate and/or dimethylallyl diphosphate, said screening comprising: (i) carrying out the steps (a) to (d) of claim 81, preferably in combination

with one of claims 82 to 85, in the presence and absence of a prospective inhibitor; (ii) detecting difference(s) in the level(s) of 1-deoxy-D-xylulose and/or 1-deoxy-D-xylulose 5-phosphate and/or 2C-methyl-D-erythritol 4-phosphate and/or 4-diphosphocytidyl-2C-methyl-D-erythritol and/or 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate and/or 2C-methyl-D-erythritol 2,4-cyclodiphosphate and/or 1-hydroxy-2-methyl-2-but enyl 4-diphosphate, notably (*E*)-1-hydroxy-2-methyl-2-but enyl 4-diphosphate and/or isopentenyl diphosphate and/or dimethylallyl diphosphate; between the presence and absence of a prospective inhibitor and (iii) correlating said detected difference(s) with the presence or absence of an above-defined inhibition.

87. The process according to claim 74 or 86, wherein said detecting of step (ii) is done by HPLC and/or NMR spectroscopy.
88. Vektor comprising a sequence coding for one of the recombinant endowments as defined in one of claims 75 to 80.
89. A process for the chemical preparation of a compound of formula I or a salt thereof:

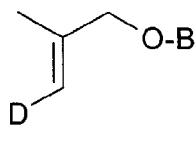


wherein A represents $-\text{CH}_2\text{OH}$ and R^1 and R^2 are different from each other and one of R^1 and R^2 is hydrogen and the other is $-\text{CH}_2\text{-O-PO(OH)-O-PO(OH)}_2$, $-\text{CH}_2\text{-O-PO(OH)}_2$ or $-\text{CH}_2\text{-OH}$ by the following steps:

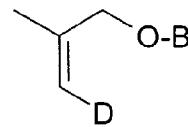
- (a) converting a compound of the following formula (II):



wherein B is a protective group into a compound of the following formula (III) or (IV):



(III)



(IV)

by a Wittig or Horner reagent, wherein the group D is a precursor group convertible reductively to a -CH₂-OH group;

- (b) reductively converting group D to a -CH₂-OH group;
- (c) optionally converting group -CH₂-OH obtained in step (b) into -CH₂-O-PO(OH)-O-PO(OH)₂ or -CH₂-O-PO(OH)₂ or salts thereof in a manner known per se;
- (d) optionally conversion to a desired salt;
- (e) removing the protective group B.

90. The process according to claim 89, wherein said protective group B forms an acetal together with the remaining moiety of the compound of formula (II).

91. The process according to claim 89 or 90, wherein said protective group B is a 2-tetrahydropyranyl group.

92. The process according to one of claims 89 to 91, wherein group D is an alkoxy carbonyl group.

93. The process according to one of claims 89 to 92, wherein said reduction of step (b) is performed with a metal hydride, notably an aluminium hydride or a boron hydride.

94. The process according to one of claims 89 to 93, wherein step (c) comprises converting said -CH₂-OH group to a -CH₂-halide group.

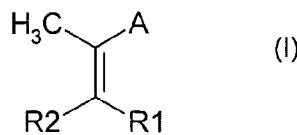
95. The process according to one of claims 89 to 94, wherein step (c) comprises reacting said -CH₂-OH group with a sulfonic acid halogenide, notably tosyl chloride.

96. The process of one of claims 89 to 95, wherein step (c) comprises a reaction with phosphoric acid or diphosphoric acid or a salt thereof.

97. The process of one of claims 89 to 96, wherein steps (a) to (c) are carried out in aprotic solvents.

98. The process of one of claims 89 to 97, wherein step (e) is carried out by acid hydrolysis.

99. A process for the chemical preparation of a compound of formula I or a salt thereof:



wherein A represents -CH₂OH or -CHO, R¹ is hydrogen, and R² is -CH₂-O-PO(OH)-O-PO(OH)₂, -CH₂-O-PO(OH)₂ or -CH₂-OH by the following steps:

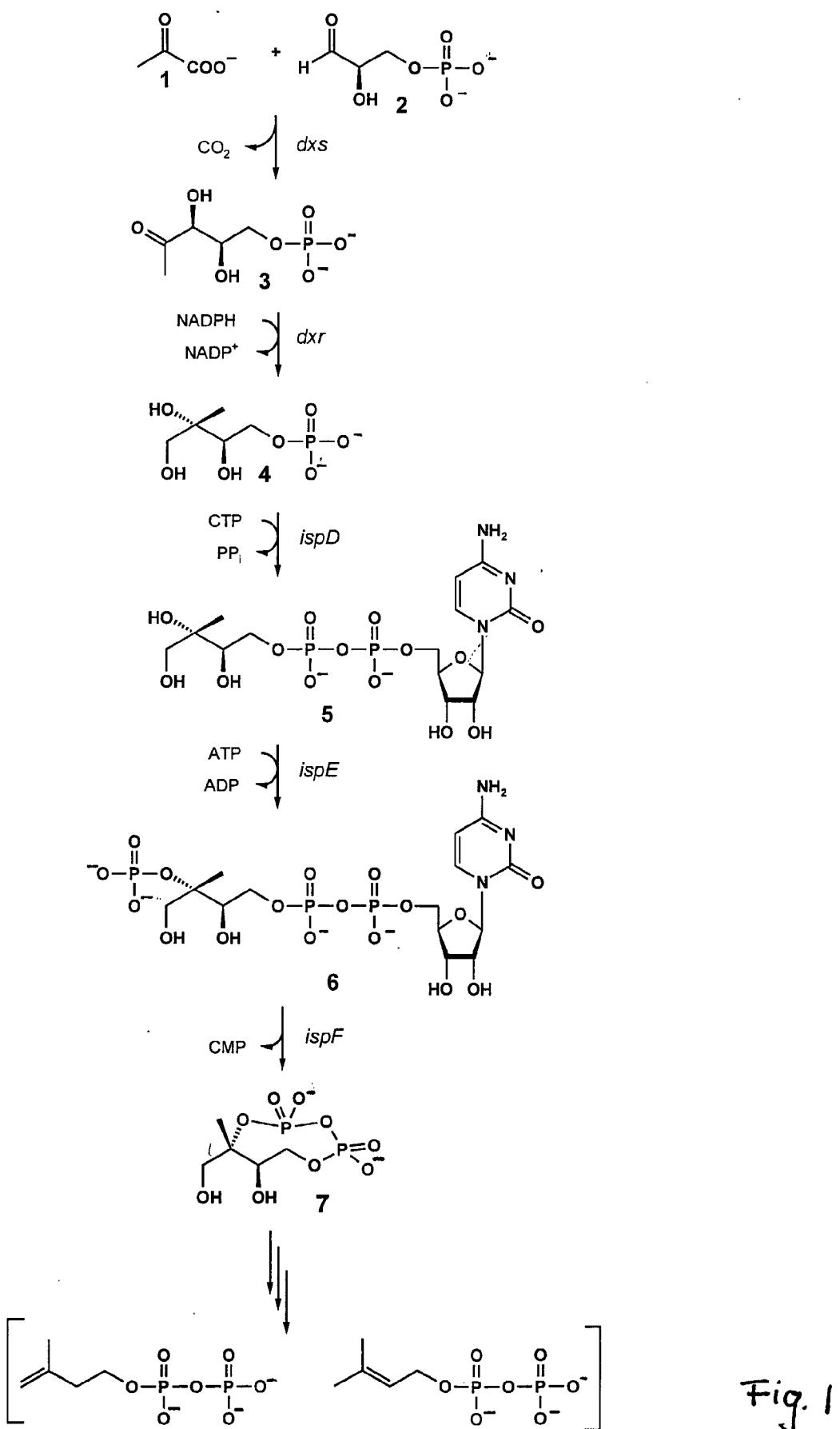
- (a) converting 2-methyl-2-vinyl-oxiran into 4-chloro-2-methyl-2-buten-1-al;
- (b) converting 4-chloro-2-methyl-2-buten-1-al to its acetal;
- (c) substituting the chlorine atom in the product of step (b) by a hydroxyl group, a phosphate group or a pyrophosphate group;
- (d) hydrolysing the acetal obtained in step (c) to produce an aldehyde group;
- (e) optionally converting the aldehyde group of the product of step (d) to a -CH₂OH group.

100. The process of claim 99, wherein step (a) is carried out in the presence of CuCl₂.

101. The process of claim 99 or 100, wherein step (b) is carried out in the presence of an ortho alkyl ester of formic acid.

102. The process of one of claims 99 to 101, wherein R² is -CH₂-O-PO(OH)-O-PO(OH)₂ or -CH₂-O-PO(OH)₂ and step (c) is carried out by reacting the product of step (b) with a tetra-alkylammonium pyrophosphate or a tetra-alkylammonium phosphate, respectively, in a polar aprotic solvent.

103. The process of one of claims 99 to 102, wherein step (e) is performed with an alkali metal borohydride in aqueous solution.



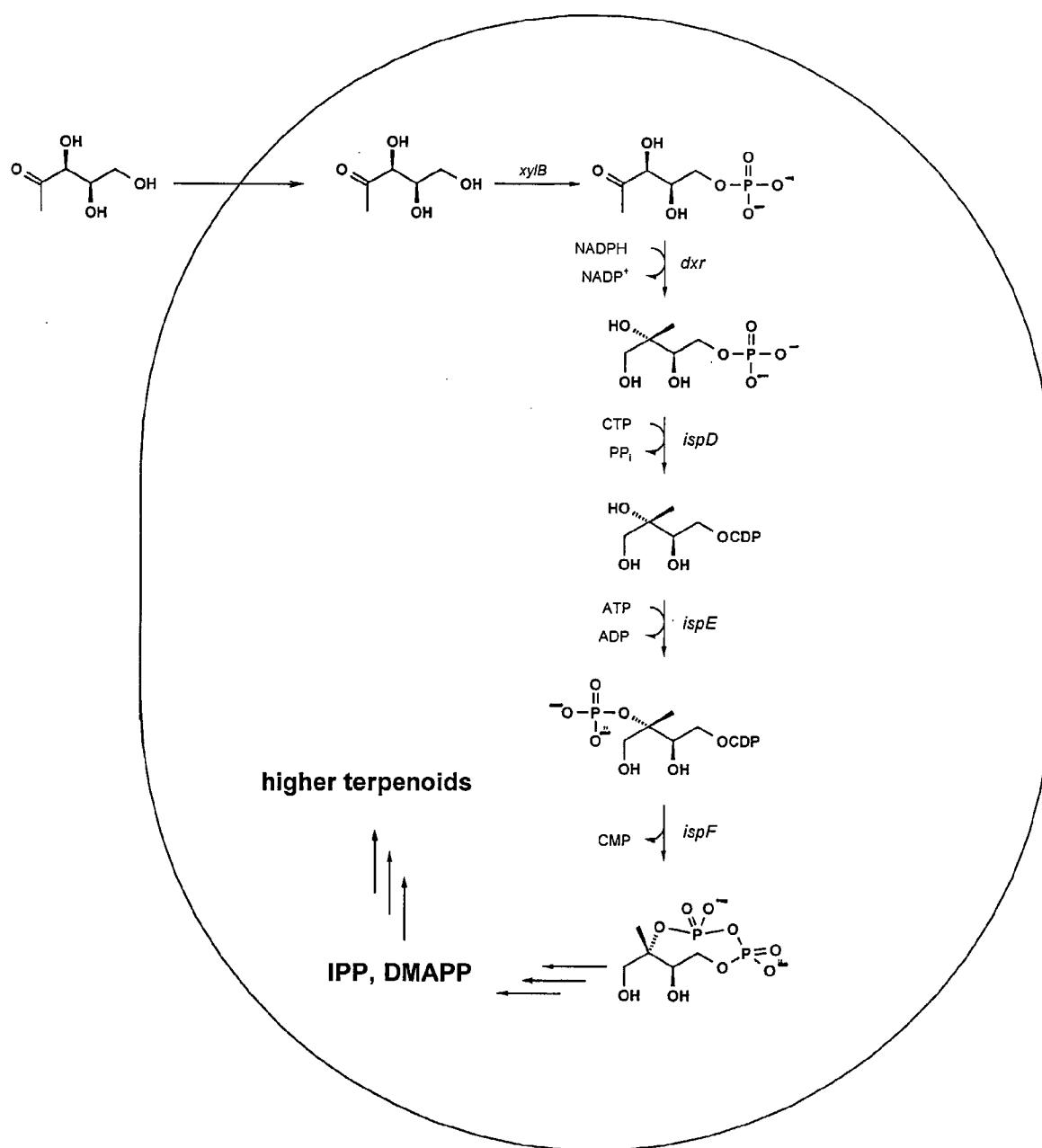


Fig. 2

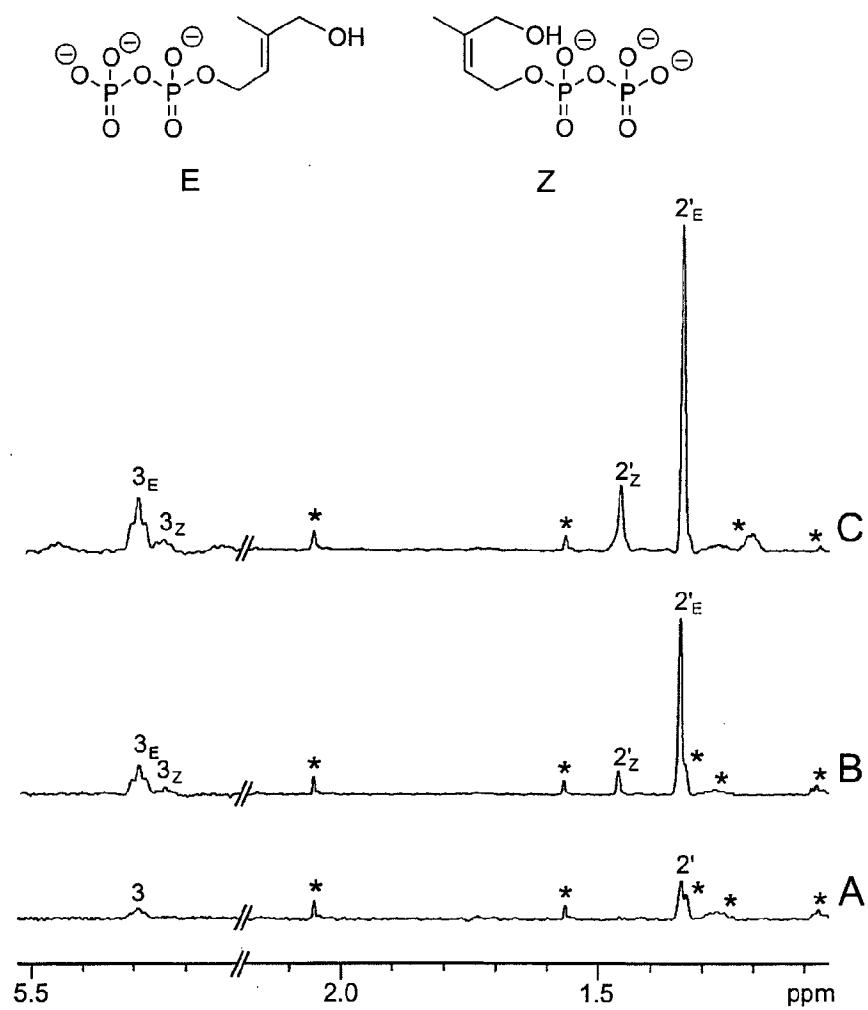


Fig. 3

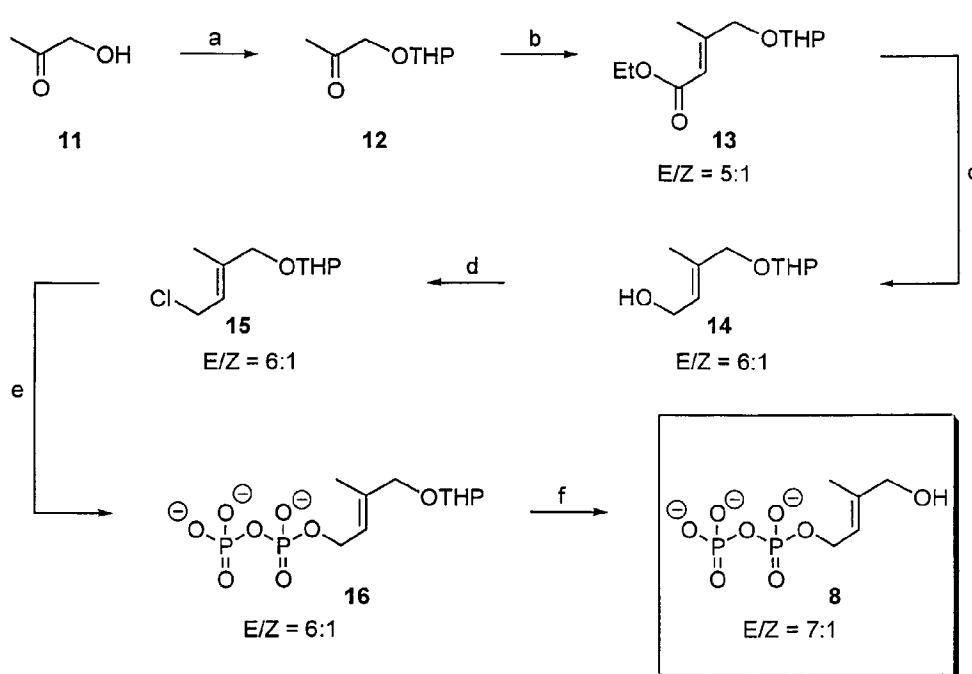


Fig. 4

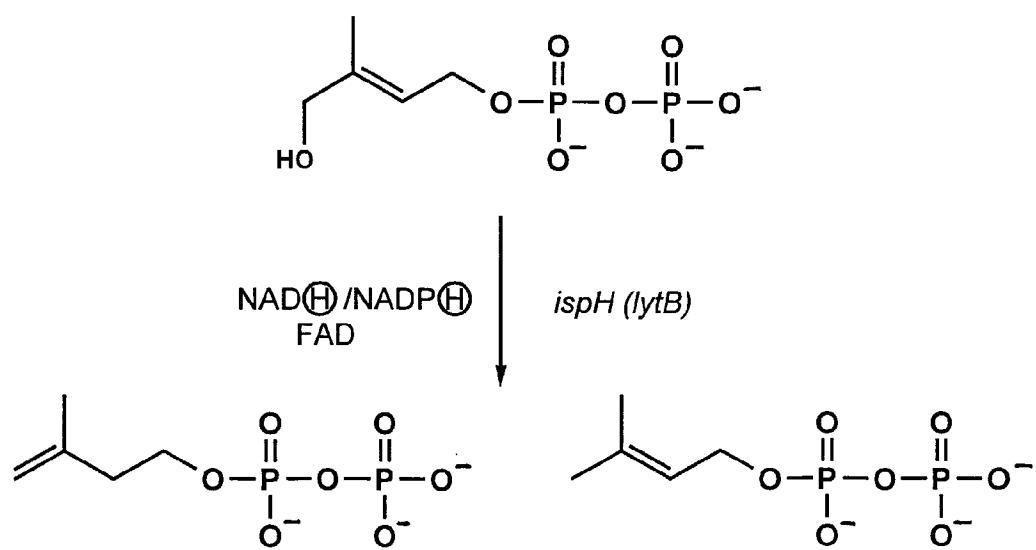


Fig. 5

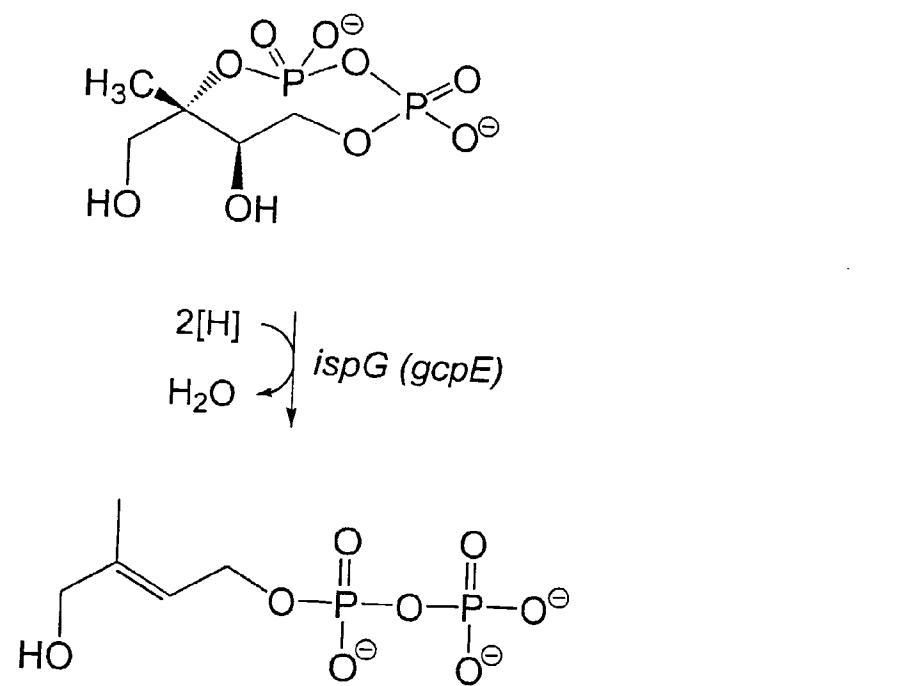


Fig. 6

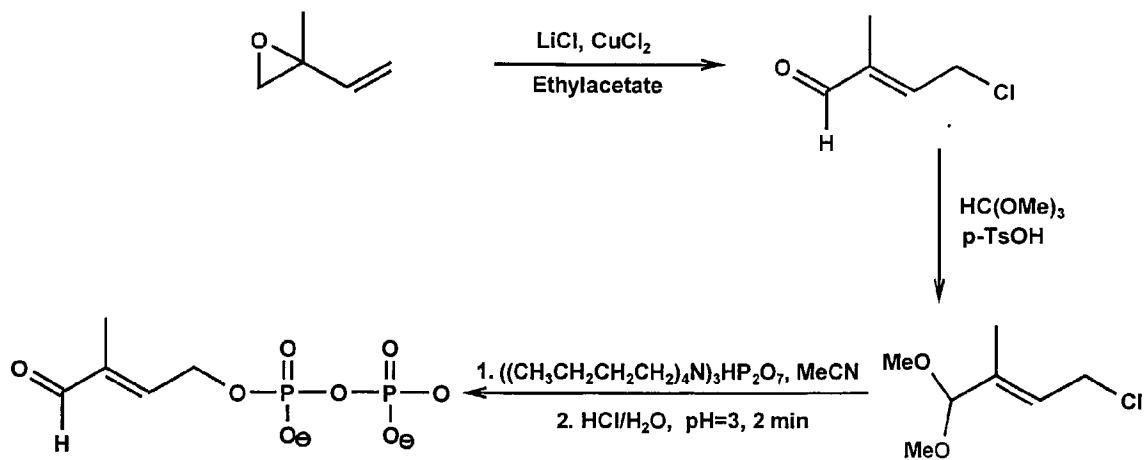


Fig. 7

Annex A

DNA sequence of the vector construct pBSxylBdxr

ID PBSXYLBDXR PRELIMINARY; DNA; 5628 BP.

SQ SEQUENCE 5628 BP; 1378 A; 1374 C; 1552 G; 1324 T; 0 OTHER;

GTGGCACTTT TCGGGGAAAT GTGCGCGAA CCCCTATTG TTTATTTTC TAAATACATT
 CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAAT GCTTCATAA TATTGAAAAA
 GGAAGAGTAT GAGTATTCAA CATTCCGTG TCGCCCTTAT TCCCTTTTT GCGGCATT
 GCCTTCTGT TTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT
 TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT
 TTCGCCCGA AGAACGTTT CCAATGATGA GCACCTTTAA AGTTCTGCTA TGTGGCGCG
 TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTG CGGCATACAC TATTCTCAGA
 ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
 GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TCGGGCCAAC TTACTTCTGA
 CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTGCA CAACATGGGG GATCATGTAA
 CTCGCCTTGA TCGTTGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
 CCACGATGCC TGTAGCAATG GCAACAAACGT TGGCCTAACT ATTAACTGGC GAAACTACTTA
 CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC
 TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCGGGTGAGC
 GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG
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 TAGGTGCCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT
 AGATTGATTT AAAACTTCAT TTTTAATTAA AAAGGATCTA GGTGAAGATC CTTTTGATA
 ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCGTAG
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 CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA
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TAACCACAC ACCCGCCGCG CTTAATGCGC CGCTACAGGG CGCGTCAG

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Annex B

DNA sequence of the vector construct pBSxylBdxrispD

ID PBSXYLBDXRISPD PRELIMINARY; DNA; 6354 BP.

SQ SEQUENCE 6354 BP; 1539 A; 1573 C; 1753 G; 1489 T;

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Annex C**DNA sequence of the vector construct pBScyclo**

| | | | | | | |
|----|----------|--------------|---------|----------|---------|---------|
| ID | PBSCYCLO | PRELIMINARY; | DNA; | 7691 BP. | | |
| SQ | SEQUENCE | 7691 BP; | 1844 A; | 1888 C; | 2148 G; | 1811 T; |

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TCCGTGAGAC GCCATTGCGC GATTTGGCAA CAATGACGCC GGATCAAGCC TGCCGTCATC
CGAACTGGTC GATGGGGCGT AAAATTCTG TCGATTCGGC TACCATGATG AACAAAGGTC
TGGAATACAT TGAAGCGCGT TGGCTGTTA ACGCCAGCGC CAGCCAGATG GAAGTGCTGA
TTCACCCGCA GTCAGTGATT CACTCAATGG TGCCTATCA GGACGGCAGT GTTCTGGCGC
AGCTGGGGA ACCGGATATG CGTACGCCA TTGCCACAC CATGGCATGG CCGAATCGCG
TGAACCTCTGG CGTGAAGCCG CTCGATTTT GCAAACTAAG TGCCTTGACA TTTGCCGAC
CGGATTATGA TCGTTATCCA TGCCTGAAAC TGGCGATGGA GGCGTTGAA CAAGGCCAGG
CAGCGACGAC AGCATTGAAT GCCGCAAACG AAATCACCCTG TGCTGCTTT CTTGCGAAC
AAATCCGCTT TACGGATATC GCTGCGTTGA ATTATCCGT ACTGGAAAAA ATGGATATGC
GCGAACACA ATGTGTGGAC GATGTGTTAT CTGTTGATGC GAACGCGCGT GAAGTCGCA
GAAAAGAGGT GATGCGTCTC GCAAGCTGAG TCGACGAGGA GAAATTAACC ATGGCAACCA
CTCATTGGA TGTTTGCCTC GTGGTTCCGG CGGCCGGATT TGGCGTCGA ATGCAAACGG
AATGTCTAA GCAATATCTC TCAATCGTA ATCAAACCAT TCTTGAAACAC TCGGTGCATG
CGCTGCTGGC GCATCCCCGG GTGAAACGTG TCGTCATTGC CATAAGTCCT GGCGATAGCC
GTTTGACACA ACTTCCTCTG GCGAATCATC CGCAAATCAC CGTTGTAGAT GGCGGTGATG
AGCGTGCCTA TTCCGTGCTG GCAGGTCTGA AAGCCGCTGG CGACGCGCAG TGGTATTGG
TGCATGACGC CGCTCGTCCT TGTTGACATC AGGATGACCT CGCGCGATTG TTGGCGTTGA
GCGAAACAG CGGCACGGGG GGGATCCTCG CCGCACCAAGT GCGCGATACT ATGAAACGTG
CCGAACCGGG CAAAAATGCC ATTGCTCATA CCGTTGATCG CAACGGCTTA TGGCACGCGC
TGACGCGCA ATTTCCTCCCT CGTGAGCTGT TACATGACTG TCTGACGCC GCTCTAAATG
AAGGCGCGAC TATTACCGAC GAAGCCTCGG CGCTGGAATA TTGCGGATTC CATCCTCAGT
TGGTCGAAGG CCGTGCCTG AACATTAAAG TCACCGCCTC GGAAGATTTG GCACTGGCCG
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GGGTTATACC CTTGGCAACG TCGATGTCAC TATCATCGCT CAGGCACCGA AGATGTTGCC
GCACATTCCA CAAATGCGCG TGTTTATTGC CGAAGATCTC GGCTGCCATA TGGATGATGT
TAACGTGAA GCCACTACTA CGGAAAAACT GGGATTTACC GGACGTGGGG AAGGGATTGC
CTGTGAAGCG GTGGCGCTAC TCATTAAGGC AACAAAATGA CTCGAGGAGG AGAAATTAAC
CATGCGGACA CAGTGGCCCT CTCCGGCAA ACTTAATCTG TTTTATACA TTACCGGTCA
GCGTGCCTG GGTTACCA CGCTGCAAAC GCTGTTTCAG TTTCTTGATT ACGCGACAC
CATCAGCATT GAGCTTCGTG ACGATGGGA TATTGCTCTG TTAACGCCCG TTGAAGGCGT

GGAACATGAA GATAACCTGA TCGTCGCGC AGCGCGATTG TTGATGAAAA CTGCGGCAGA
CAGCGGGCGT CTTCCGACGG GAAGCGGTGC GAATATCAGC ATTGACAAGC GTTTGCCGAT
GGCGGGCGGT CTCGGCGGTG GTTCATCCAA TGCGCGACG GTCCTGGTGG CATTAAATCA
TCTCTGGCAA TGCGGGCTAA GCATGGATGA GCTGGCGGAA ATGGGGCTGA CGCTGGCGC
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CAGCACATCC CCCCTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC GATGCCCTT
CCCAACAGTT GCGCAGCCTG AATGGCGAAT GGAAATTGTA AGCGTTAATA TTTTGTAAA
ATTCGCGTTA AATTTTGTT AAATCAGCTC ATTTTTAAC CAATAGGCCG AAATCGGCAA
AATCCCTTAT AAATCAAAAG AATAGACCGA GATAGGGTTG AGTGTGTTTC CAGTTGGAA
CAAGAGTCCA CTATTAAGA ACGTGGACTC CAACGTCAA GGGCGAAAAA CCGTCTATCA
GGCGATGGC CCACTACGTG AACCATCACC CTAATCAAGT TTTTGGGGT CGAGGTGCCG
TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT AGAGCTTGAC GGGGAAAGCC
GGCGAACGTG GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC
AAAGTGTAGCG GTCACGCTGC GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA
GGCGCGTCA G

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Annex D

DNA sequence of the vector construct pACYCgcpE

ID PACYCGCPE PRELIMINARY; DNA; 5109 BP.

SQ SEQUENCE 5109 BP; 1194 A; 1365 C; 1324 G; 1226 T; 0 OTHER;

GAATTCCGGA TGAGCATTCA TCAGGCGGGC AAGAATGTGA ATAAAGGCCG GATAAAACTT
 GTGCTTATTT TTCTTACGG TCTTTAAAAA GGCGTAATA TCCAGCTGAA CGGTCTGGTT
 ATAGGTACAT TGAGCAACTG ACTGAAATGC CTCAAAATGT TCTTACGAT GCCATTGGGA
 TATATCAACG GTGGTATATC CAGTGATTT TTTCTCCATT TTAGCTTCCT TAGCTCCTGA
 AAATCTCGAT AACTCAAAAATACGCCGG TAGTGATCTT ATTTCATTAT GGTGAAAGTT
 GGAACCTCTT ACGTGCCGAT CAACGTCTCA TTTCGCCAA AAGTTGGCCC AGGGCTTCCC
 GGTATCAACA GGGACACCCAG GATTATTTA TTCTGCGAAG TGATCTTCG TCACAGGTAT
 TTATTGGCG CAAAGTGCCTG CGGGTGTG TGCCAACCTTA CTGATTTAGT GTATGATGGT
 GTTTTGAGG TGCTCCAGTG GCTTCTGTTT CTATCAGCTG TCCCTCCTGT TCAGCTACTG
 ACGGGGTGGT GCGTAACGGC AAAAGCACCG CGGACATCA GCGCTAGCGG AGTGTATACT
 GGCTTACTAT GTGGCACTG ATGAGGGTGT CAGTGAAAGTG CTTCATGTGG CAGGAGAAAA
 AAGGCTGCAC CGGTGCGTCA GCAGAATATG TGATACAGGA TATATTCCGC TTCCTCGCTC
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 GGAGATTTC CAGGAAGATGC CAGGAAGATA CTTAACAGGG AAGTGAGAGG GCCGGCGCAA
 AGCCGTTTTT CCATAGGCTC CGCCCCCCTG ACAAGCATCA CGAAATCTGA CGCTCAAATC
 AGTGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTCCCCCT GGCGGCTCCC
 TCGTGCCTC TCCTGTTCCCT GCCTTTCGGT TTACCGGTGT CATTCCGCTG TTATGGCCGC
 GTTTGTCTCA TTCCACGCCT GACACTCAGT TCCGGTAGG CAGTCGCTC CAAGCTGGAC
 TGTATGCACG AACCCCCCGT TCAGTCCGAC CGCTGCGCCT TATCCGGTAA CTATCGCTT
 GAGTCCAACC CGGAAAGACA TGCAAAAGCA CCACTGGCAG CAGCCACTGG TAATTGATTT
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 CGAAAAACCG CCCTGCAAGG CGGTTTTTC GTTTTCAGAG CAAGAGATTA CGCGCAGACC
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 TCACCCCTGGA TGCTGTAGGC ATAGGCTTGG TTATGCCGGT ACTGCCGGC CTCTTGCAGGG
 ATATCGTCCA TTCCGACAGC ATCGCCAGTC ACTATGGCGT GCTGCTAGCG CTATATCGGT
 TGATGCAATT TCTATGCGCA CCCGTTCTCG GAGCACTGTC CGACCGCTTT GGCGCCGCC
 CAGTCCTGCT CGCTTCGCTA CTTGGAGCCA CTATCGACTA CGCGATCATG GCGACCACAC
 CGTCCTGTG GATCCGAGGA GAAATTAACC ATGCATAACC AGGCTCCAAT TCAACGTAGA

AAATCAACAC GTATTTACGT TGGGAATGTG CCGATTGGCG ATGGTGCTCC CATGCCGTA
CAGTCCATGA CCAATACGCG TACGACAGAC GTCGAAGCAA CGGTCAATCA AATCAAGGCG
CTGGAACGCG TTGGCGCTGA TATCGTCGCT GTATCCGTAC CGACGATGGA CGCGGCAGAA
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TATCGCATTG CGCTGAAAGT AGCGGAATAC GGCGTCGATT GTCTGCGTAT TAACCCCTGGC
AATATCGGT A ATGAAGAGCG TATTCGCATG GTGGTTGACT GTGCGCGGA TAAAAACATT
CCGATCCGTA TTGGCGTTAA CGCCGGATCG CTGGAAAAAG ATCTGCAAGA AAAGTATGGC
GAACCGACGC CGCAGGGCGT GCTGGAATCT GCCATGCGTC ATGTTGATCA TCTCGATCGC
CTGAACCTCG ATCAGTTCAA AGTCAGCGTG AAAGCGTCTG ACGTCTTCCT CGCTGTTGAG
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GGTGGTGC GCAAGCGGGGC AGTAAAATCC GCCATTGGTT TAGGTCTGCT GCTGTCGAA
GGCATCGCG ACACGCTGCG CGTATCGCTG GCGGCCGATC CGGTCGAAGA GATCAAAGTC
GGTTTCGATA TTTGAAATC GCTGCGTATC CGTCGCGAG GGATCAACTT CATCGCCTGC
CCGACCTGTT CGCGTCAGGA ATTTGATGTT ATCGGTACGG TTAACCGCCT GGAGCAACGC
CTGGAAAGATA TCATCACTCC GATGGACGTT TCGATTATCG GCTGCGTGGT GAATGGCCCA
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GAAGATGGCG TGCGCAAAGA CCGTCTGGAC AACAAACGATA TGATCGACCA GCTGGAAGCA
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CGATGATCGG CCTGTCGCTT GCGGTATTCG GAATCTTGCA CGCCCTCGCT CAAGCCTTCG
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CCGACGCGCT GGGCTACGTC TTGCTGGCGT TCGCGACGCG AGGCTGGATG GCCTTCCCCA
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GGCAGGTAGA TGACGACCAT CAGGGACAGC TTCAAGGATC GCTCGCGGCT CTTACCAGCC
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GGAACGGTT GGCATGGATT GTAGGCGCCG CCCTATACCT TGTCTGCTC CCCCGTTG
GTCGCGGTGC ATGGAGCCGG GCCACCTCGA CCTGAATGGA AGCCGGCGGC ACCTCGCTAA
CGGATTCAACC ACTCCAAGAA TTGGAGCCAA TCAATTCTTG CGGAGAACTG TGAATGCGCA
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GACCCGGCTA GGCTGGCGGG GTTGCCTTAC TGGTTAGCAG AATGAATCAC CGATACGCGA
GCGAACGTGA AGCGACTGCT GCTGAAACAC GTCTGCGACC TGAGCAACAA CATGAATGGT
CTTCGGTTTC CGTGTTCGTT AAAGTCTGGA AACCGGAAAG TCCCCTACGT GCTGCTGAAG
TTGCCCGCAA CAGAGAGTGG AACCAACCGG TGATACCACG ATACTATGAC TGAGAGTCAA
CGCCATGAGC GGCCTCATT TCTATTCTGA GTTACAACAG TCCGCACCGC TGTCCGGTAG

CTCCTTCCGG TGGGCGCGGG GCATGACTAT CGTCGCCGCA CTTATGACTG TCTTCTTTAT
CATGCAACTC GTAGGACAGG TGCCGGCAGC GCCCAACAGT CCCCCGGCCA CGGGGCCTGC
CACCATACCC ACGCCGAAAC AAGCGCCCTG CACCATTATG TTCCGGATCT GCATCGCAGG
ATGCTGCTGG CTACCCCTGTG GAACACCTAC ATCTGTATTA ACGAAGCGCT AACCGTTTT
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GCCTGAGCAA ACTGGCCTCA GGCATTTGAG AAGCACACGG TCACACTGCT TCCGGTAGTC
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AGGCGTAGCA CCAGGCAGTT AAGGGCACCA ATAACGCCT TAAAAAAATT ACGCCCCGCC
CTGCCACTCA TCGCAGTACT GTTGTAAATTC ATTAAGCATT CTGCGACAT GGAAGCCATC
ACAGACGGCA TGATGAACCT GAATGCCAG CGGCATCAGC ACCTTGTGCGC CTTGCGTATA
ATATTTGCCA ATGGTAAAAA CGGGGGCGAA GAAGTTGTCC ATATTGGCCA CGTTAAATC
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TTTAGGGAAA TAGGCCAGGT TTTCACCGTA ACACGCCACA TCTTGCAGAT ATATGTGTAG
AAAACGCCGG AAATCGTCGT GGTATTCACT CCAGAGCGAT GAAAACGTTT CAGTTGCTC
ATGGAAAACG GTGTAACAAG GGTGAACACT ATCCCATATC ACCAGCTCAC CGTCTTCAT
TGCCATACG

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Annex E

DNA sequence of the plasmid pBScaro14

ID PBSCARO14 PRELIMINARY; DNA; 7494 BP.SQ SEQUENCE 7494 BP;
 1722 A; 1935 C; 2026 G; 1811 T;

GTGGCACTTT TCAGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTC TAAATACATT
 CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAAT GCTTCATAA TATTGAAAAAA
 GGAAGAGTAT GAGTATTCAA CATTCCGTG TCGCCCTTAT TCCCTTTTT GCAGCATT
 GCCTTCCTGT TTTGCTCAC CCAGAAACGC TGGTGAAGT AAAAGATGCT GAAGATCAGT
 TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT
 TTCGCCCCGA AGAACGTTT CCAATGATGA GCACCTTTAA AGTTCTGCTA TGTGGCCGG
 TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTG CGGCATACAC TATTCTCAGA
 ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
 GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TCGGGCAAC TTACTCTGA
 CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTGCA CAACATGGGG GATCATGTAA
 CTCGCCTTGA TCGTTGGAA CGGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
 CCACGATGCC TGTAGCAATG GCAACAAACGT TCGCACAACT ATTAACTGGC GAACTACTTA
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CTGCGCATCT CTGGCAGCGC CCGCTCTAGC GCCATGTGCA CCTCGAGGGG GGGCCCGGTA
CCCAATTGCG CCTATAGTGA GTCGTATTAC GCGCGCTCAC TGGCCGTGTT TTTACAACGT
CGTGACTGGG AAAACCCCTGG CGTTACCCAA CTTAATCGCC TTGCAAGCACA TCCCCCTTTC
GCCAGCTGGC GTAATAGCGA AGAGGCCCCG ACCGATCGCC CTTCCCAACA GTTGCAGC
CTGAATGGCG AATGGAAATT GTAAGCGTTA ATATTTGTT AAAATTGCG TAAATTTTT
GTTAAATCAG CTCATTTTT AACCAATAGG CCGAAATCGG CAAAATCCCT TATAAATCAA
AAGAATAGAC CGAGATAGGG TTGAGTGTG TTCCAGTTG GAACAAGAGT CCACTATTAA
AGAACGTGGA CTCCAACGTC AAAGGGCGAA AAACCGTCTA TCAGGGCGAT GGCCCACCTAC
GTGAACCATC ACCCTAATCA AGTTTTTGG GGTGAGGTG CCGTAAAGCA CCAAATCGGA
ACCCTAAAGG GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA
AGGAAGGGAA GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGTAA GCGGTCACGC
TGCCTGTAAC CACCACACCC GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TCAG

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Annex F

DNA sequence of the vector construct pACYCcaro14

ID PACYCCARO14 PRELIMINARY; DNA; 8547 BP.

SQ SEQUENCE 8547 BP; 1884 A; 2296 C; 2279 G; 2088 T; 0 OTHER;
 GAATTCCGGA TGAGCATTCA TCAGGCAGGC AAGAATGTGA ATAAAGGCCG GATAAAACTT
 GTGCTTATTT TTCTTTACGG TCTTTAAAAA GGCGTAATA TCCAGCTGAA CGGTCTGGTT
 ATAGGTACAT TGAGCAACTG ACTGAAATGC CTCAAAATGT TCTTTACGAT GCCATTGGGA
 TATATCAACG GTGGTATATC CAGTGATTT TTCTCCATT TTAGCTTCCT TAGCTCCTGA
 AAATCTCGAT AACTCAAAAA ATACGCCCG TAGTGATCTT ATTCATTAT GGTGAAAGTT
 GGAACCTCTT ACGTGCCGAT CAACGTCTCA TTTTCGCCAA AAGTTGGCCC AGGGCTTCCC
 GGTATCAACA GGGACACCAG GATTTATTTA TTCTGCGAAG TGATCTTCG TCACAGGTAT
 TTATTCCGCG CAAAGTGCCT CGGGTGATGC TGCCAACCTA CTGATTTAGT GTATGATGGT
 GTTTTTGAGG TGCTCCAGTG GCTTCTGTTT CTATCAGCTG TCCCTCCTGT TCAGCTACTG
 ACGGGGTGGT GCGTAACGGC AAAAGCACCG CGGGACATCA GCGCTAGCGG AGTGTATACT
 GGCTTACTAT GTTGGCACTG ATGAGGGTGT CAGTGAAGTG CTTCATGTGG CAGGAGAAAA
 AAGGCTGCAC CGGTGCGTCA GCAGAATATG TGATACAGGA TATATTCCGC TTCCCTCGCTC
 ACTGACTCGC TACGCTCGGT CGTTGACTG CGGCGAGCGG AAATGGCTTA CGAACGGGGC
 GGAGATTTC CTTGAAGATGC CAGGAAGATA CTTAACAGGG AAGTGAGAGG GCGCGGGCAA
 AGCCGTTTT CCATAGGCTC CGCCCCCCTG ACAAGCATCA CGAAATCTGA CGCTCAAATC
 AGTGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTCCCCCT GGCGGCTCCC
 TCGTGCCTC TCCGTGTTCT GCCTTTCGGT TTACCGGTGT CATTCCGCTG TTATGGCCGC
 GTTTGTCTCA TTCCACGCCT GACACTCAGT TCCGGGTAGG CAGTTCGCTC CAAGCTGGAC
 TGTATGCACG AAACCCCCCGT TCAGTCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT
 GAGTCCAACC CGGAAAGACA TGCAAAAGCA CCACTGGCAG CAGCCACTGG TAATTGATTT
 AGAGGAGTTA GTCTTGAAGT CATGCGCCGG TTAAGGCTAA ACTGAAAGGA CAAGTTTG
 TGACTGCGCT CCTCCAAGCC AGTTACCTCG GTTCAAAGAG TTGGTAGCTC AGAGAACCTT
 CGAAAAACCG CCCTGCAAGG CGGTTTTTC GTTTTCAGAG CAAGAGATTA CGCGCAGACC
 AAAACGATCT CAAGAAGATC ATCTTATTAA TCAGATAAAA TATTTCTAGA TTTCAGTGCA
 ATTTATCTCT TCAAATGTAG CACCTGAAGT CAGCCCCATA CGATATAAGT TGTAATTCTC
 ATGTTTGACA GCTTATCATC GATAAGCTTT AATGCGGTAG TTTATCACAG TTAAATTGCT
 AACGCAGTCA GGCACCGTGT ATGAAATCTA ACAATGCGCT CATCGTCATC CTCGGCACCG
 TCACCCCTGGA TGCTGTAGGC ATAGGCTTGG TTATGCCGGT ACTGCCGGC CTCTTGCGGG
 ATATCGTCCA TTCCGACAGC ATGCCAGTC ACTATGGCGT GCTGCTAGCG CTATATGCCT
 TGATGCAATT TCTATGCGCA CCCGTTCTCG GAGCACTGTC CGACCGCTTT GGCCGCCGCC
 CAGTCCTGCT CGCTTCGCTA CTTGGAGCCA CTATCGACTA CGCGATCATG GCGACCACAC
 CCGTCCTGTG GATCCCCCGG GCTGCAGGAA TTGCCGTAAA TGATCCGTT TATAAGGACA
 GCGCGAATGA CGGTCTGCGC AAAAAAACAC GTTCATCTCA CTCGGCATGC TGCAGGACAG
 TTACTGGCTG ATATTGATCG ACGCCTTGAT CAGTTATTGC CGTGGAGGG AGAACGGGAT
 GTTGTGGGTG CGCGCATGCG TGAAGGTGCG CTGGCACCAG GAAAACGTAT TCGCCCCATG
 TTGCTGTTGC TGACCGCCCG CGATCTGGGT TGCCTGTC GGCATGACGG ATTACTGGAT
 TTGGCCTGTG CGGTGGAAAT GGTCCACGCG GCTTCGCTGA TCCTTGACGA TATGCCCTGC
 ATGGACGATG CGAACGCTGCG GCGCGGACGC CCTACCATTC ATTCTCATTA CGGAGAGCAT
 GTGGCAATAC TGGCGGCGGT TGCCTGCTG AGTAAAGCCT TTGGCGTAAT TGCGATGCA
 GATGGCCTCA CGCCGCTGGC AAAAAATCGG GCGGTTCTG AACTGTCAA CGCCATCGGC
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 GCTGAAGCTA TTTTGATGAC GAATCACTTT AAAACCAAGCA CGCTGTTTG TGCCTCCATG
 CAGATGGCCT CGATTGTTGC GAATGCCCTC AGCGAAGCGC GTGATTGCCT GCATGTTTT
 TCACITGATC TTGGTCAGGC ATTTCAACTG CTGGACGATT TGACCGATGG CATGACCGAC
 ACCGGTAAGG ATAGCAATCA GGACGCCGGT AAATCGACGC TGGTCAATCT GTTAGGCCCC
 AGGGCGGTTG AAGAACGTCT GAGACAACAT CTTCAGCTTG CCAGTGAGCA TCTCTCTGCG
 GCCTGCCAAC ACGGGCACGC CACTCAACAT TTTATTCAAGG CCTGGTTTGA CAAAAAAACTC
 GCTGCCGTCA GTTAAGCTTA TGTGCACCGG TCAGCCTGTC TTAAGTGGGA GCGGCTATGC
 AACCGCATTAA TGATCTGATT CTCGTGGGGG CTGGACTCGC GAATGGCCTT ATCGCCCTGC

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CGGCAAATTG AGCACTGAGC GTGGGCTTCC AGGCAGTTAT TGGCCAGGAA TGGCGATTGA
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TGTGGTTCAT ACCTATCGCG ACCTGTTAAG CCAGCACCC GCGCGGTAA AGCAGTCCAA
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TGACGAAATT TTTAATCATG ATGGCCTCGC AGAGGACTTC TCACTTTATC TGACGCGCC
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CAATACAGCC AACTGGATGA TACGCTCGC TATTGCTATC ACGTTGCAGG CGTTGTCGGC
TTGATGATGG CGCAAATCAT GGGCGTCGG GATAACGCCA CGCTGGACCG CGCTGTGAC
CTTGGGCTGG CATTTCAGTT GACCAATATT GCTCGCGATA TTGTGGACGA TGCCATGCG
GGCGCTGTT ATCTGCCGGC AAGCTGGCTG GAGCATGAAG GTCTGAACAA AGAGAATTAT
CGGGCACCTG AAAACCGTCA GGCGCTGAGC CGTATGCC GTCGTTGGT GCAGGAAGCA
GAACCTTACT ATTTGTCTGC CACAGCCGGC CTGGCAGGGT TGCCCCCTGCG TTCCGCCTGG

GCAATCGCTA CGCGGAAGCA GGTTTACCGG AAAATAGGTG TCAAAGTTGA ACAGGCCGGT
CAGCAAGCCT GGGATCAGCG GCAGTCAACG ACCACGCCCG AAAAATTAAC GCTGCTGCTG
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TCGCGGCTCT TACCAAGCCTA ACTTCGATCA CTGGACCGCT GATCGTCACG GCGATTATG
CCGCCTCGGC GAGCACATGG AACGGGTTGG CATGGATTGT AGGCAGGCC CTATACCTTG
TCTGCCTCCC CGCGTTGCGT CGCGGTGCAT GGAGCCGGC CACCTCGACC TGAATGGAAG
CCGGCGGCAC CTCGCTAACG GATTCACAC TCCAAGAATT GGAGCCAATC AATTCTTGCG
GAGAACTGTG AATGCGAAA CCAACCCCTG GCAGAACATA TCCATCGCGT CGCCCATCTC
CAGCAGCCGC ACGGCGCGA TCTCGGGCAG CGTGGGTCC TGGCCACGGG TGCGCATGAT
CGTGCCTCTG TCGTTGAGGA CCCGGCTAGG CTGGCGGGGT TGCCCTTACTG GTTAGCAGAA
TGAATCACCG ATACCGCAGC GAACGTGAAG CGACTGCTGC TGCAAAACGT CTGGCACCTG
AGCAACAAACA TGAATGGTCT TCGGTTTCCG TGTTCGTTAA AGTCTGGAAA CGCGGAAGTC
CCCTACGTGC TGCTGAAGTT GCCCCAACAA GAGAGTGGAA CCAACCGGTG ATACCACGAT
ACTATGACTG AGAGTCAACG CCATGAGCGG CCTCATTCT TATTCTGAGT TACAACAGTC
CGCACCGCTG TCCGGTAGCT CCTTCCGGTG GGCGCGGGC ATGACTATCG TCGCCGCACT
TATGACTGTC TTCTTTATCA TGCAACTCGT AGGACAGGTG CGGGCAGCGC CCAACAGTCC
CCCGGCCACG GGGCCTGCCA CCATACCCAC GCGAAACAA GCGCCCTGCA CCATTATGTT
CCGGATCTGC ATCGCAGGAT GCTGCTGGCT ACCCTGTGGA ACACCTACAT CTGTATTAAC
GAAGCGCTAA CGGTTTTTAT CAGGCTCTGG GAGGCAGAAT AAATGATCAT ATCGTCAATT
ATTACCTCCA CGGGGAGAGC CTGAGCAAC TGGCCTCAGG CATTTGAGAA GCACACGGTC
ACACTGCTTC CGGTAGTCAA TAAACCGTA ACCAGCAAT AGACATAAGC GGCTATTTAA
CGACCCGTCC CTGAACCGAC GACCGGGTCG AATTGCTTT CGAATTCTG CCATTCACTCC
GCTTATTATC ACTTATTCA GCGTAGCACC AGGCCTTAA GGGCACCAAT AACTGCCTTA
AAAAAATTAC GCCCCGCCCT GCCACTCATC GCAGTACTGT TGTAATTCAAT TAAGCATTCT
GCCGACATGG AAGCCATCAC AGACGGCATG ATGAACCTGA ATCGCCAGCG GCATCAGCAC
CTTGTGCCT TCGGTATAAT ATTTGCCCAT GGTGAAAACG GGGCGAAGA AGTTGTCCAT
ATTGGCCACG TTTAAATCAA AACTGGTGA ACTCACCCAG GGATTGGCTG AGACGAAAAAA
CATATTCTCA ATAAACCCCTT TAGGAAATA GGCAAGGTT TCACCGTAAC ACGCCACATC
TTGCGAATAT ATGTGTAGAA ACTGCCGGAA ATCGTCGTGG TATTCACTCC AGAGCGATGA
AACGTTCA GTTGCTCAT GGAAAACGGT GTAACAAGGG TGAACACTAT CCCATATCAC
CAGCTCACCG TCTTCATTG CCATACG

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Annex G

DNA and corresponding amino acid sequence of the *ispG* (formely *gcpE*) gene of *Escherichia coli*

| | | | | | |
|--|----|----|----|----|----|
| 10 | 20 | 30 | 40 | 50 | 60 |
| ATGCATAACCAGGCTCCAATTCAACGTAGAAAATCAACACGTATTTACGTTGGGAATGTG | | | | | |
| M H N Q A P I Q R R K S T R I Y V G N V | | | | | |
| 70 80 90 100 110 120 | | | | | |
| CCGATTGGCGATGGTGCCTCCATGCCGTACAGTCCATGACCAATACGCGTACGACAGAC | | | | | |
| P I G D G A P I A V Q S M T N T R T T D | | | | | |
| 130 140 150 160 170 180 | | | | | |
| GTCGAAGCAACGGTCAATCAAATCAAGGCCTGGAACGCGTGGCGCTGATATCGTCCGT | | | | | |
| V E A T V N Q I K A L E R V G A D I V R | | | | | |
| 190 200 210 220 230 240 | | | | | |
| GTATCCGTACCGACGATGGACCGGGCAGAACGCTTCAAACCTCATCAAACAGCAGGTTAAC | | | | | |
| V S V P T M D A A E A F K L I K Q Q V N | | | | | |
| 250 260 270 280 290 300 | | | | | |
| GTGCCGCTGGTGGCTGACATCCACTTCGACTATCGCATTGCGCTGAAAGTAGCGGAATAC | | | | | |
| V P L V A D I H F D Y R I A L K V A E Y | | | | | |
| 310 320 330 340 350 360 | | | | | |
| GGCGTCGATTGTCGCTATTAAACCTGGCAATATCGGTAAATGAAGAGCGTATTGCGATG | | | | | |
| G V D C L R I N P G N I G N E E R I R M | | | | | |
| 370 380 390 400 410 420 | | | | | |
| GTGGTTGACTGTGCGCGATAAAAACATTCCGATCCGTATTGGCGTTAACGCCGGATCG | | | | | |
| V V D C A R D K N I P I R I G V N A G S | | | | | |
| 430 440 450 460 470 480 | | | | | |
| CTGGAAAAAGATCTGCAAGAAAAGTATGGCGAACCGACGCCGCAGGCCTGCTGGAATCT | | | | | |
| L E K D L Q E K Y G E P T P Q A L L E S | | | | | |
| 490 500 510 520 530 540 | | | | | |
| GCCATGCGTCATGTTGATCATCTCGATGCCCTGAACCTCGATCAGTTCAAAGTCAGCGTG | | | | | |
| A M R H V D H L D R L N F D Q F K V S V | | | | | |

| | | | | | |
|--|------|------|------|------|------|
| 550 | 560 | 570 | 580 | 590 | 600 |
| AAAGCGTCTGACGTCTTCCTCGCTGTTGAGTCTTATCGTTGCTGGCAAAACAGATCGAT | | | | | |
| K A S D V F L A V E S Y R L L A K Q I D | | | | | |
| 610 | 620 | 630 | 640 | 650 | 660 |
| CAGCCGTTGCATCTGGGGATCACCGAAGCCGGTGGTGCAGCGCAGCGGGGAGTAAATCC | | | | | |
| Q P L H L G I T E A G G A R S G A V K S | | | | | |
| 670 | 680 | 690 | 700 | 710 | 720 |
| GCCATTGGTTTAGGTCTGCTGCTGTCTGAAGGCATGGCGACACGCTGCGCGTATCGCTG | | | | | |
| A I G L G L L S E G I G D T L R V S L | | | | | |
| 730 | 740 | 750 | 760 | 770 | 780 |
| GCGGCCGATCCGGTCGAAGAGATCAAAGTCGGTTTCGATATTTGAAATCGCTGCGTATC | | | | | |
| A A D P V E E I K V G F D I L K S L R I | | | | | |
| 790 | 800 | 810 | 820 | 830 | 840 |
| CGTTCGCGAGGGATCAACTCATCGCCTGCCGACCTGTTCGCGTCAGGAATTGATGTT | | | | | |
| R S R G I N F I A C P T C S R Q E F D V | | | | | |
| 850 | 860 | 870 | 880 | 890 | 900 |
| ATCGGTACGGTTAACCGCCTGGAGCAACGCCCTGGAAGATATCATCACTCCGATGGACGTT | | | | | |
| I G T V N A L E Q R L E D I I T P M D V | | | | | |
| 910 | 920 | 930 | 940 | 950 | 960 |
| TCGATTATCGGCTCGTGGTGAATGGCCAGGTGAGGCCTGGTTCTACACTCGCGTC | | | | | |
| S I I G C V V N G P G E A L V S T L G V | | | | | |
| 970 | 980 | 990 | 1000 | 1010 | 1020 |
| ACCGGGCGCAACAAGAAAAGCGGCCTCTATGAAGATGGCGTGCCTGAAAGACCGTCTGGAC | | | | | |
| T G G N K K S G L Y E D G V R K D R L D | | | | | |
| 1030 | 1040 | 1050 | 1060 | 1070 | 1080 |
| AACAAACGATATGATCGACCAAGCAGCTGGAAGCACGCATTGCGAAAGCCAGTCAGCTGGAC | | | | | |
| N N D M I D Q L E A R I R A K A S Q L D | | | | | |
| 1090 | 1100 | 1110 | | | |
| GAAGCGCGTCGAATTGACGTTCAAGCAGGTTGAAAAATAA | | | | | |
| E A R R I D V Q Q V E K - | | | | | |

Annex H

DNA sequence of the vector construct pBScyclogcpE

| | | | | | | | |
|----|-----------|--------------|---------|----------|---------|---------|----------|
| ID | PBSCYCLOG | PRELIMINARY; | DNA; | 8823 BP. | | | |
| SQ | SEQUENCE | 8823 BP; | 2123 A; | 2169 C; | 2468 G; | 2063 T; | 0 OTHER; |

GTGGCACTTT TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTC TAAATACATT
 CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAAT GCTTCAATAA TATTGAAAAA
 GGAAGAGTAT GAGTATTCAA CATTCCGTG TCGCCCTTAT TCCCTTTTT GCGGCATTT
 GCCTTCCGTG TTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT
 TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT
 TTCGCCCCGA AGAACGTTT CCAATGATGA GCACCTTTAA AGTTCTGCTA TGTGGCGCGG
 TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTG CCGCATACAC TATTCTCAGA
 ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
 GAGAATTATG CAGTGCCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA
 CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTGCA CAACATGGGG GATCATGTAA
 CTCGCCTTGA TCGTTGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
 CCACGATGCC TGTAGCAATG GCAACAAACGT TGCGCAAACAT ATTAACTGGC GAACTACTTA
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 TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA
 TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAAGACCA AGTTTACTCA TATATACTTT
 AGATTGATTT AAAACTTCAT TTTAATTAA AAAGGATCTA GGTGAAGATC CTTTTGATA
 ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG
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 CAAAAAAACC ACCGCTACCA GCGGTGGTTT GTTTGCCTGGA TCAAGAGCTA CCAACTCTTT
 TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC
 CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA
 TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG TTGGACTCAA
 GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGTTCG TGACACACAGC
 CCAGCTTGG ACGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA
 GCGCCACGCT TCCCGAAGGG AGAAAGGCCG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA
 CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTAT AGTCCCTGTCG
 GGTTTGCCTA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC
 TATGGAAAAA CGCCAGCAAC GCGGCCTTT TACGGTTCTC GGCTTTTGC TGGCCTTTTG
 CTCACATGTT CTTCTCTGCG TTATCCCCGT ATTCTGTGGA TAACCGTATT ACCGCCTTTG
 AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTC GTGAGCGAGG
 AAGCGGAAGA CGGCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCAATTAT
 GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC GCAATTATG
 TGAGTTAGCT CACTCATTAG GCACCCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT
 TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAA CAGCTATGAC CATGATTACG
 CCAAGCGCGC AATTAACCCCT CACTAAAGGG AACAAAAGCT GGAGCTCCAC CGCGGGAGGA
 GAAATTAACC ATGCATAACC AGGCTCCAAT TCAACGTAGA AAATCAACAC GTATTTACGT
 TGGGAATGTG CCGATTGGCG ATGGTGCCTC CATGCCGTG CAGTCATGA CCAATACGCG
 TACGACAGAC GTCGAAGCAA CGGTCAATCA AATCAAGGCG CTGGAACGCG TTGGCGCTGA
 TATCGTCCGT GTATCCGTAC CGACGATGGA CGCGGCAGAA GCGTTCAAAC TCATCAAACA
 GCAGGTTAAC GTGCCGCTGG TGGCTGACAT CCACCTCGAC TATCGCATTG CGCTGAAAGT
 AGCGGAATAC GGCCTCGATT GTCTGCGTAT TAACCCCTGGC AATATCGGTG ATGAAGAGCG
 TATTGCGATG GTGGTTGACT GTGCGCGCGA TAAAAACATT CCGATCGGTG TTGGCGTTAA
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GCTGGAATCT GCCATGCGTC ATGTTGATCA TCTCGATCGC CTGAACCTCG ATCAGTTCAA
AGTCAGCGTG AAAGCGTCTG ACGTCTTCC CGCTGTTGAG TCTTATCGTT TGCTGGCAAA
ACAGATCGAT CAGCGTTGC ATCTGGGAT CACCGAAGCC GGTGGTGCAGC GCAGCGGGGC
AGTAAAATCC GCCATTGGTT TAGGTCTGCT GCTGTCTGAA GGCATCGCG ACACGCTGCG
CGTATCGCTG GCGGCCGATC CGGTGCAAGA GATCAAAGTC GGTTTCGATA TTTTGAAATC
GCTCGTATC CGTTCGCGAG GGATCAAATT CATCGCCTGC CCGACCTGTT CGCGTCAGGA
ATTTGATGTT ATCGGTACGG TTAACGCGCT GGAGCAACGC CTGGAAGATA TCATCACTCC
GATGGACGTT TCGATTATCG GCTCGTGGT GAATGGCCCA GGTGAGGCCG TGTTTCTAC
ACTCGCGTC ACCGGCGGCA ACAAGAAAAG CGGCCTCTAT GAAGATGCCG TGCGCAAAGA
CCGTCTGGAC AACAAACGATA TGATCGACCA GCTGGAAGCA CGCATTCTG CGAAAGCCAG
TCAGCTGGAC GAAGCGCGTC GAATTGACGT TCAGCAGGTT GAAAATAAG CGGCCGCTCT
AGAACTAGTG GATCCCCCGG GCTGCAGGAA TTCGAGGAGA AATTAACCAT GTATATCGGG
ATAGATCTTG GCACCTCGGG CGTAAAAGTT ATTTTGCCTCA ACGAGCAGGG TGAGGTGGTT
GCTGCGAAA CGGAAAAGCT GACCGTTTCG CGCCCGCATC CACTCTGGTC GGAACAAGAC
CCGGAACAGT GGTGGCAGGC AACTGATCGC GCAATGAAAG CTCTGGCGA TCAGCATTCT
CTGCAGGACG TTAAAGCATT GGGTATTGCC GGCCAGATGC ACGGAGCAAC CTTGCTGGAT
GCTCAGCAAC GGGTGTACG CCCTGCCATT TTGTGGAACG ACGGGCGCTG TGCGCAAGAG
TGCACTTGC TCCAAGCGCG AGTTCCGCAA TCGCGGGTGA TTACCGGCAA CCTGATGATG
CCCGGATTAA CTGCGCCTAA ATTGCTATGG GTTCAGCGGC ATGAGCCGA GATATTCCGT
CAAATCGACA AAGTATTATT ACCGAAAGAT TACTTGCCTC TGCGTATGAC GGGGGAGTTT
GCCAGCGATA TGTCTGACGC AGCTGGCACC ATGTGGCTGG ATGTCGCAA CGGTGACTGG
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GGGTTCTTAA GCAAGCCAGA AAGCGCCGTA CATACTTTT GCCATGCGCT ACCCGAACGT
TGGCATTAA TGTCTGTGAT GCTGAGTGCA GCGTCGTGTC TGGATTGGC CGCGAAATTA
ACCGGCCTGA GCAATGTCCC AGCTTTAATC GCTGCAGCTC AACAGGCTGA TGAAAGTGC
GAGCCAGTTT GGTTTCTGCC TTATCTTCC GGCGAGCGTA CGCCACACAA TAATCCCCAG
GCGAAGGGGG TTTCTTTGG TTTGACTCAT CAAACATGGCC CCAATGAACG GCGCGAGCA
GTGCTGGAAG GCGTGGGTTA TGCCTGGCA GATGGCATGG ATGTCGTGCA TGCCTGCGGT
ATTAAACCGC AAAGTGTAC GTTGATTGGG GGCGGGCGC GTAGTGAAGTA CTGGCGTCAG
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TATCAGCCAC GACGAGAAAC GTTCCGTGCG CTCTATCAGC AACCTCTGCC ATTAATGGCG
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CTTGAGCAA ATGGCGTGGT GTCCATTAA CTTACCGGGT CTGGTGGCCC TTTCGTTGAG
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CAGTCAGTGA TTCACTCAAT GGTGCGCTAT CAGGACGGCA GTGTTCTGGC GCAGCTGGGG
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GATCGTTATC CATGCCCTGAA ACTGGCGATG GAGGCGTTCG AACAAAGGCCA GGCAGCGACG
ACAGCATTGA ATGCCGCAA CGAAATCAC CTTGCTGCTT TTCTGCGCA ACAAAATCCGC
TTTACGGATA TCGCTGCGTT GAATTATCC GTACTGGAAA AAATGGATAT GCGCGAACCA

CAATGTGTGG ACGATGTGTT ATCTGTTGAT GCGAACGCGC GTGAAGTCGC CAGAAAAGAG
GTGATGCGTC TCGCAAGCTG AGTCGACGAG GAGAAATTAA CCATGGCAAC CACTCATTG
GATGTTGCG CCGTGGTTCC GGCGGCCGGA TTTGGCGTC GAATGCAAAC GGAATGTCCT
AAGCAATATC TCTCAATCGG TAATCAAACC ATTCTTGAAC ACTCGGTGCA TGCGCTGCTG
GCGCATCCCC GGGTGAACG TGTCGTCATT GCCATAAGTC CTGGCGATAG CCGTTTGCA
CAACTTCTC TGGCGAATCA TCCGCAAATC ACCGTTGTAG ATGGCGGTGA TGAGCGTGC
GATTCCGTGC TGGCAGGTCT GAAAGCCGCT GGCGACGCGC AGTGGGTATT GGTGCATGAC
GCCGCTCGTC CTTGTTGCA TCAGGATGAC CTCGCGCGAT TGTTGGCGTT GAGCGAAACC
AGCCGCACGG GGGGGATCCT CGCCGCACCA GTGCGCGATA CTATGAAACG TGCCGAACCG
GGCAAAAATG CCATTGCTCA TACCGTTGAT CGCAACGGCT TATGGCACGCG GCTGACGCC
CAATTTTCC CTCGTGAGCT GTTACATGAC TGTCCTGACGC GCGCTCTAAA TGAAGGCCG
ACTATTACCG ACGAACGCCTC GGCGCTGGAA TATTGCGGAT TCCATCCTCA GTTGGTCGAA
GGCGTGCAG ATAACATTAA AGTCACCGC CCGGAAGATT TGGCACTGGC CGAGTTTAC
CTCACCCGAA CCATCCATCA GGAGAAATACA TAATGCGAAT TGGACACGGT TTTGACGTAC
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GATTGCTGGC GCATTCTGAT GGCGACGGTGG CGCTCCATGC GTTGACCGAT GCATTGCTTG
GCGCGGCCGC GCTGGGGGAT ATCGGCAAGC TGTTCCCGGA TACCGATCCG GCATTAAAG
GTGCCGATAG CCGCGAGCTG CTACGCGAAG CCTGGCGTCG TATTCAAGCG AAGGGTTATA
CCCTTGGCAA CGTCGATGTC ACTATCATCG CTCAGGCACC GAAGATGTTG CCGCACATT
CACAAATGCG CGTGTGTTATT GCGGAAGATC TCGGCTGCCA TATGGATGAT GTTAACGTGA
AAGCCACTAC TACGGAAAAA CTGGGATTAA CGGACAGTGG GGAAGGGATT GCCTGTGAAG
CGGTGGCGCT ACTCATTAAG GCAACAAAAT GACTCGAGGA GGAGAAATTA ACCATGCGGA
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AAGATAACCT GATCGTTCGC GCAGCGCGAT TGTGATGAA AACTGCGGCA GACAGGGGC
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TGGATCCGCC AGAGAAAGTGG TATCTGGTGG CGCACCCCTGG TGTAAGTATT CCGACTCC
TGATTTTAA AGATCCTGAA CTCCCGCGCA ATACGCCAA AAGGTCAATA GAAACGTTGC
TAAAATGTGA ATTCAAGCAAT GATTGCCAGG TTATCGCAAG AAAACGTTT CGCGAGGTT
ATGCGGTGCT TTCTGGCTG TTAGAATACG CCCGTCGCG CCTGACTGGG ACAGGGGC
GTGTCTTGC TGAATTTGAT ACAGAGTCTG AAGCCCGCA GGTGCTAGAG CAAGCCCCGG
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CCCCCTTCG CCAGCTGGCG TAATAGCGAA GAGGCCGCA CGATGCCCT TTCCAACAG
TTGCGCAGCC TGAATGGCGA ATGGAAATTG TAAGCGTTAA TATTTGTTA AAATTGCG
TAAATTTTG TTAAATCAGC TCATTTTTA ACCAATAGGC CGAAATCGGC AAAATCC
ATAAAATCAA AGAATAGACC GAGATAGGGT TGAGTGTGTT TCCAGTTGG AACAAGAGTC
CACTATTAA GAACTGGAC TCCAACGTCA AAGGGCGAAA AACCGTCTAT CAGGGCGATG
GCCCACTACG TGAACCATCA CCCTAATCAA GTTTTTGGG GTCGAGGTGC CGTAAAGCAC
TAAATCGGAA CCCTAAAGGG AGCCCCGAT TTAGAGCTTG ACGGGAAAG CGGGCGAAC
TGGCGAGAAA GGAAGGGAAAG AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG GCAAGTGTAG
CGGTACGCT GCGCGTAACC ACCACACCCG CCGCGCTTAA TGCGCCGCTA CAGGGCGCGT
CAG

Annex I

DNA sequence of the vectorconstruct pACYClytBgcP

ID PACYCGCLY PRELIMINARY; DNA; 5793 BP.

SQ SEQUENCE 5793 BP; 1375 A; 1506 C; 1543 G; 1369 T; 0 OTHER;

GAATTCGGATGAGCATTCA TCAGGGCGGGC AAGAAATGTGA ATAAAGGCCG GATAAAACTT
 GTGCTTATTT TTCTTACGG TCTTTAAAAA GGCGTAATA TCCAGCTGAA CGGTCTGGTT
 ATAGGTACAT TGAGCAACTG ACTGAAATGC CTCAAAATGT TCTTTACGAT GCCATTGGGA
 TATATCAACG GTGGTATATC CAGTGATTTT TTCTCCATT TTAGCTTCCCT TAGCTCCTGA
 AAATCTCGAT AACTAAAAA ATACGCCGG TAGTGATCTT ATTCATTAT GGTGAAAGTT
 GGAACCTCTT ACGTGCCGAT CAACGCTCTA TTTTCGCCAA AAGTTGGCCC AGGGCTTCCC
 GGTATCAACA GGGACACCAAG GATTATTTA TTCTGCGAAG TGATCTTCCG TCACAGGTAT
 TTATTGGCG CAAAGTGCCT CGGGTGATGC TGCCAACCTA CTGATTTAGT GTATGATGGT
 GTTTTGAGG TGCTCCAGTG GCTTCTGTTT CTATCAGCTG TCCCTCCGTG TCAGCTACTG
 ACGGGGTGGT GCGTAACGGC AAAAGCACCG CGGACATCA GCGCTAGCGG AGTGTATACT
 GGCTTACTAT GTTGGCACTG ATGAGGGTGT CAGTGAAGTG CTTCATGTGG CAGGAGAAAA
 AAGGCTGCAC CGGTGCGTCA GCAGAATATG TGATACAGGA TATATTCCGC TTCCTCGCTC
 ACTGACTCGC TACGCTCGGT CGTTGACTG CGCGAGCGG AAATGGCTTA CGAACGGGGC
 GGAGATTTC TGGAAAGATGC CAGGAAGATA CTTAACAGGG AAGTGAGAGG GCCGCGGCAA
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 AGTGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGCGGCTCCC
 TCGTGCCTC TCCTGTTCCCT GCCTTCGGT TTACCGGTGT CATTCCGCTG TTATGGCCGC
 GTTTGCTCTA TTCCACGCTC GACACTCAGT TCCGGTAGG CAGTTGCTC CAAGCTGGAC
 TGTATGCACG AACCCCCCGT TCAGTCCGAC CGTGCCTCGT TATCCGGTAA CTATCGTCTT
 GAGTCCAACC CGGAAAGACA TGCAAAAGCA CCACTGGCAG CAGCCACTGG TAATTGATT
 AGAGGAGTTA GTCTTGAAGT CATGCGCCGG TTAAGGCTAA ACTGAAAGGA CAAGTTTGG
 TGACTGCGCT CCTCCAAGGC AGTTACCTCG GTTCAAAGAG TTGGTAGCTC AGAGAACCTT
 CGAAAAAACCG CCCTGCAAGG CGGTTTTTC GTTTTCAGAG CAAGAGATTA CGCGCAGACC
 AAAACGATCT CAAGAAGATC ATCTTATTA TCAGATAAAA TATTTCTAGA TTTCACTGCA
 ATTTATCTCT TCAAATGTAG CACCTGAAGT CAGCCCCATA CGATATAAGT TGTAATTCTC
 ATGTTGACA GCTTATCATC GATAAGCTTT AATGCGGTAG TTATCACAG TAAATTGCT
 AACGCAGTCA GGCACCGTGT ATGAAATCTA ACAATGCGCT CATCGTCATC CTCGGCACCG
 TCACCCCTGGA TGCTGTAGGC ATAGGCTTGG TTATGCCGGT ACTGCCGGC CTCTGCGGG
 ATATCGCCA TTCCGACAGC ATGCCAGTC ACTATGCCGT GCTGCTAGCG CTATATGCGT
 TGATGCAATT TCTATGCGCA CCCGTTCTCG GAGCACTGTC CGACCGCTT GGCCGCCGCC
 CAGCCTGCT CGCTTCGCTA CTTGGAGCCA CTATCGACTA CGCGATCATG GCGACCACAC
 CCGTCCTGTG GATCCGAGGA GAAATTAAAC ATGCATAACC AGGCTCCAAT TCAACGTAGA
 AAATCAACAC GTATTTACGT TGGGAATGTG CCGATTGGCG ATGGTGCTCC CATGCCGTA
 CAGTCCATGA CCAATACGCG TACGACAGAC GTCGAAGCAA CGGTCAATCA AATCAAGGCG
 CTGGAACGCG TTGGCGCTGA TATCGCTCGT GTATCCGTAC CGACGATGGA CGCGGCAGAA
 CGGTTCAAAC TCATCAAACA GCAGGTTAAC GTGCCGCTGG TGGCTGACAT CCACTTCGAC
 TATCGCATTG CGCTGAAAGT AGCGGAATAC GGCGTCGATT GTCTGCGTAT TAACCCTGGC
 AATATCGGTA ATGAAAGAGCG TATTCGCTATG GTGGTTGACT GTGCGCGGA TAAAACATT
 CCGATCCGTA TTGGCGTTAA CGCCGGATCG CTGAAAAAG ATCTGCAAGA AAAGTATGGC
 GAACCGACGC CGCAGGGCGT GCTGGAATCT GCCATGCGTC ATGTTGATCA TCTCGATCGC
 CTGAACCTCG ATCAGTTCAA AGTCAGCGTG AAAGCGTCTG ACGTCTTCCCT CGCTGTTGAG
 TCTTATCGTT TGCTGGCAAAC ACAGATCGAT CAGCCGTTGC ATCTGGGAT CACCGAAGCC
 GGTGGTGCAC GCAGCGGGGC AGTAAATCC GCCATTGGTT TAGGTCTGCT GCTGCTGAA
 GGCATCGCGC ACACGCTGCG CGTATCGCTG CGGGCCGATC CGGTGAGA GATCAAAGTC
 GGTTTCGATA TTTGAAATC GCTGCGTATC CGTTCGCGAG GGATCAACTT CATCGCCTGC
 CCGACCTGTT CGCGTCAGGA ATTTGATGTT ATCGGTACGG TAAACGCGCT GGAGCAACGC
 CTGGAAGATA TCATCACTCC GATGGACGTT TCGATTATCG GCTGCGTGGT GAATGGCCCA
 GGTGAGGCAC TGGTTCTAC ACTCGGGCGTC ACCGGCGGCA ACAAGAAAAG CGGCCTCTAT

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GAAAATAAG TCGACGAGGA GAAATTAAACC ATGCAGATCC TGTGGCCAA CCCCGCGTGGT
TTTGTGCCG GGGTAGACCG CGCTATCAGC ATTGTTGAAA ACGCGCTGGC CATTACGGC
GCACCGATAT ATGTCCGTCA CGAAGTGGTA CATAACCGCT ATGTGGTCGA TAGCTTGCCT
GAGCGTGGGG CTATCTTAT TGAGCAGATT AGCGAAGTAC CGGACGGCGC GATCCTGATT
TTCTCCGCAC ACGGTGTTTC TCAGGGCGTA CGTAACGAAG CAAAAGTCG CGATTGACG
GTGTTGATG CCACCTGTCC GCTGGTGACC AAAGTGCATA TGGAAAGTCGC CCGCGCCAGT
CGCCGTGGCG AAGAATCTAT TCTCATCGGT CACGCCGGC ACCCGGAAGT GGAAGGGACA
ATGGGCCAGT ACAGTAACCC GGAAGGGGG AATGATATCTGG TCGAATCGCC GGACGATGTG
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GTGGATGACA CGTCTGATGT GATCGACGCG CTGCGTAAC GCTTCCGAA AATTGTCGGT
CCGCGCAAAG ATGACATCTG CTACGCCACG ACTAACCGTC AGGAAGCGGT ACGGCCCTG
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CTGGCGGAGC TGGCCCGAGCG TATGGGCAAA CGCGCGTTTG TGATTGACGA TGCGAAAGAC
ATCCAGGAAG AGTGGGTGAA AGAGGTTAAA TGCCTCGGCG TGAATGCGGG CGCATCGGCT
CCGGATATTC TGGTGCAGAA TGTGGTGGCA CGTTTGCAGC AGCTGGGGGG TGGTGAAGCC
ATTCCGCTGG AAGGCCGTGA AGAAAACATT GTTTTCAAG TGCCGAAAGA GCTGCGTGT
GATATTGCG AAGTCGATTA ACGGCCGACG CGCTGGCTA CGTCTTGTG CGGTTGCG
CGCGAGGCTG GATGGCCTTC CCCATTATGA TTCTTCTCGC TTCCGGCGC ATCGGGATGC
CCGCGTTGCA GGCCATGCTG TCCAGGCAAG TAGATGACGA CCATCAGGG AAGCTTCAAG
GATCGCTCGC GGCTCTTACCG AGCCTAACCT CGATCACTGG ACCGCTGATC GTCACGGCGA
TTTATGCCGC CTCGGCGAGC ACATGGAACG GGTGGCATG GATTGAGGC GCCGCCCTAT
ACCTTGCTG CCTCCCCCGCG TTGCGTCGCG GTGCATGGAG CGGGGCCACC TCGACCTGAA
TGGAAAGCCGG CGGCACCTCG CTAACGGATT CACCACTCCA AGAATTGGAG CCAATCAATT
CTTGCAGGAGA ACTGTGAATG CGCAAACCAA CCCTTGGCAG AACATATCCA TCGCGTCCGC
CATCTCCAGC AGCCGCACGC GGCGCATCTC GGGCAGCGTT GGGTCTGGC CACGGGTGCG
CATGATCGT CTCTGTGCGT TGAGGACCCG GCTAGGCTGG CGGGGTTGCC TTACTGGTTA
GCAGAAATGAA TCACCGATAC GCGAGCGAAC GTGAAGCGAC TGCTGCTGCA AAACGTCTGC
GACCTGAGCA ACAACATGAA TGGTCTTCGG TTTCCGTGTT TCGTAAAGTC TGGAAACGCG
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CACGATACTA TGACTGAGAG TCAACGCCAT GAGCGGCCTC ATTTCTTATT CTGAGTTACA
ACAGTCCGCA CCGCTGTCCG GTAGCTCCTT CCGGTGGCG CGGGGCATGA CTATGTCGC
CGCACTTATG ACTGTCTTCT TTATCATGCA ACTCGTAGGA CAGGTGCCGG CAGGCCCAA
CAGTCCCCCG GCCACGGGGC CTGCCACCAT ACCCACGCCG AAACAAGCGC CCTGCACCAT
TATGTTCCGG ATCTGCATCG CAGGATGCTG CTGGCTACCC TGTGGAACAC CTACATCTGT
ATTAACGAAG CGCTAACCGT TTTTATCAGG CTCTGGAGG CAGAATAAT GATCATATCG
TCAATTATTA CCTCCACGGG GAGAGCCTGA GCAAACCTGGC CTCAGGCATT TGAGAAGCAC
ACGGTCACAC TGCTTCCGGT AGTCAATAAA CGGTAAACC AGCAATAGAC ATAAGCGGCT
ATTTAACGAC CCTGCCCTGA ACCGACGACC GGGTGAATT TGCTTCCGAA TTTCTGCCAT
TCATCCGCTT ATTATCACTT ATTCAAGCGT AGCACCCAGGC GTTAAAGGGC ACCAATAACT
GCCTTAAAAA AATTACGCC CGCCCTGCCA CTCATCGCAG TACTGTTGTA ATTCAATTAAAG
CATTCTGCCG ACATGGAAGC CATCACAGAC GGCATGATGA ACCTGAATCG CCAGGGCAT
CAGCACCTTG TCGCCTTGCAG TATAATATTT GCCCATGGTG AAAACGGGG CGAAGAAGTT
GTCCATATTG GCCACGTTA AATCAAACCT GGTGAAACTC ACCCAGGGAT TGGCTGAGAC
GAAAAACATA TTCTCAATAA ACCCTTCTAGG GAAATAGGCC AGGTTTCAC CGTAACACGC
CACATCTTGC GAATATATGT GTAGAAACTG CGGAAATCG TCGTGGTATT CACTCCAGAG
CGATGAAAAC GTTCAGTTT GCTCATGGAA AACGGTGTAA CAAGGGTGAA CACTATCCCA
TATCACCAGC TCACCGTCTT TCATTGCCAT ACG

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Annex J

A and corresponding amino acid sequence of the *ispH* (formerly *lytB*) gene from *Escherichia coli*

| | | | | | | | |
|---|-----|-----|-----|-----|-----|-----|-----|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 | |
| | | | | | | | |
| ATGCAGATCCTGTCGCCAACCGCGTGGTTTGTGCCGGGTAGACCGCGCTATCAGCATTGTTGAAAAC | | | | | | | |
| M Q I L L A N P R G F C A G V D R A I S I V E N | | | | | | | |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 | |
| | | | | | | | |
| GCGCTGGCATTACGGCGACCGATATATGTCCGTACGAAGTGGTACATAACCGCTATGTGGTCGATAGC | | | | | | | |
| A L A I Y G A P I Y V R H E V V H N R Y V V D S | | | | | | | |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 | |
| | | | | | | | |
| TTGCGTGAGCGTGGGCTATCTTATTGAGCAGATTAGCGAAGTACCGGACGGCGATCCTGATTTCTCC | | | | | | | |
| L R E R G A I F I E Q I S E V P D G A I L I F S | | | | | | | |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 | |
| | | | | | | | |
| GCACACGGTGTTCCTCAGGCGGTACGTAACGAAGCAAAAGTCGCGATTGACGGTGTGATGCCACCTGT | | | | | | | |
| A H G V S Q A V R N E A K S R D L T V F D A T C | | | | | | | |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 | 360 |
| | | | | | | | |
| CCGCTGGTGAACCAAGTGCATATGGAAGTCGCCCGCCAGTCGCCGTGGCGAAGAATCTATTCTCATCGGT | | | | | | | |
| P L V T K V H M E V A R A S R R G E E S I L I G | | | | | | | |
| 370 | 380 | 390 | 400 | 410 | 420 | 430 | |
| | | | | | | | |
| CACGCCGGCACCCCGGAAGTGGAAAGGGACAATGGGCCAGTACAGTAACCGGAAGGGGAATGTATCTGGTC | | | | | | | |
| H A G H P E V E G T M G Q Y S N P E G G M Y L V | | | | | | | |
| 440 | 450 | 460 | 470 | 480 | 490 | 500 | |
| | | | | | | | |
| GAATCGCCGGACGATGTGTGGAAACTGACGGTCAAAACGAAGAGAAGCTCTCCTTATGACCCAGACCG | | | | | | | |
| E S P D D V W K L T V K N E E K L S F M T Q T T | | | | | | | |
| 510 | 520 | 530 | 540 | 550 | 560 | 570 | |
| | | | | | | | |
| CTGTCGGTGGATGACAGCTCTGATGTGATCGACGCCGTGGTAAACGCTTCCGAAAATTGTCGGTCCCGC | | | | | | | |
| L S V D D T S D V I D A L R K R F P K I V G P R | | | | | | | |
| 580 | 590 | 600 | 610 | 620 | 630 | 640 | |
| | | | | | | | |
| AAAGATGACATCTGCTACGCCACGACTAACCGTCAGGAAGCGGTACGCCCTGGCAGAACAGGGGAAGTT | | | | | | | |
| K D D I C Y A T T N R Q E A V R A L A E Q A E V | | | | | | | |
| 650 | 660 | 670 | 680 | 690 | 700 | 710 | 720 |
| | | | | | | | |
| GTGTTGGTGGTCGGTTCGAAAAACTCTCCAACCTCCAACCGCTGGCGGAGCTGGCCAGCGTATGGCAA | | | | | | | |
| V L V V G S K N S S N S N R L A E L A Q R M G K | | | | | | | |
| 730 | 740 | 750 | 760 | 770 | 780 | 790 | |
| | | | | | | | |
| CGCGCGTTTGATTGACGATGCGAAAGACATCCAGGAAGAGTGGTGAAAGAGGTTAAATGCGTCGGCGTG | | | | | | | |
| R A F L I D D A K D I Q E E W V K E V K C V G V | | | | | | | |
| 800 | 810 | 820 | 830 | 840 | 850 | 860 | |
| | | | | | | | |
| ACTGCGGGCGCATCGGCTCCGGATATTCTGGTGCAGAATGTGGTGCACGTTGCAGCAGCTGGCGGTGGT | | | | | | | |
| T A G A S A P D I L V Q N V V A R L Q Q L G G G | | | | | | | |
| 870 | 880 | 890 | 900 | 910 | 920 | 930 | |

GAAGCCATTCCGCTGGAAGGCCGTGAAGAAAACATTGTTTCGAAGTGCCGAAAGAGCTGCGTGTGATATT
E A I P L E G R E E N I V F E V P K E L R V D I
940 950
CGTGAAGTCGATTAA
R E V D -

Annex K

DNA Sequence of the plasmid construct pBScyclogcpElytB2

ID PBSXICH2 PRELIMINARY; DNA; 9795 BP.

SQ SEQUENCE 9795 BP; 2351 A; 2401 C; 2770 G; 2273 T; 0 OTHER;

GTGGCACTT TCGGGGAAAT GTGCGCGGAA CCCCTATTG TTTATTTTC TAAATACATT
 CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAAT GCTTCAATAA TATTGAAAAA
 GGAAGAGTAT GAGTATTCAA CATTCCGTG TCGCCCTTAT TCCCTTTT GCAGCATT
 GCCTTCTGT TTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT
 TGGGTGACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT
 TTCGCCCGA AGAACGTTT CCAATGATGA GCACTTTAA AGTTCTGCTA TGTTGCGCGG
 TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTGCG CCGCATACAC TATTCTCAGA
 ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA
 GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA
 CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTGCA CAACATGGGG GATCATGTAA
 CTCGCCCTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
 CCACGATGCC TGTAGCAATG GCAACAAACGT TGCGCAAACACT ATTAACACTGGC GAACTACTTA
 CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAAGTT GCAGGACCAC
 TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA GCCGGTGAGC
 GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG
 TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA
 TAGGTGCCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT
 AGATTGATTT AAAACTTCAT TTTTAATTAA AAAGGATCTA GGTGAAGATC CTTTTGATA
 ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA GACCCCGTAG
 AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTCTGCG CGTAATCTGC TGCTTGCAAA
 CAAAAAAACC ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTT
 TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC
 CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA
 TCCTGTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGT TCTTACCGGG TTGGACTCAA
 GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC GGGGGGTTCG TGACACACAGC
 CCAGCTTGGA GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA
 GCGCCACGCT TCCCAGAAGGG AGAAAGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA
 CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCGTG GTATCTTTAT AGTCCCTGTCG
 GGTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG GGGCGGAGCC
 TATGGAAAAA CGCCAGCAAC CGGGCTTTT TACGGTTCCCT GGCCTTTGCG TGGCCTTTG
 CTCACATGTT CTTTCTGCG TTATCCCCGT ATTCTGTGGA TAACCGTATT ACCGCTTTG
 AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG
 AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC GCGTTGGCCG ATTCAATTAA
 GCAGCTGGCA CGACAGGTTT CCCGACTGGA AAGCGGGCAG TGAGCGCAAC GCAATTAAATG
 TGAGTTAGCT CACTCATTAG GCACCCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT
 TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG
 CCAAGCGCGC AATTAAACCT CACTAAAGGG AACAAAAGCT GGAGCTCCAC CGCGGGAGGA
 GAAATTAAAC ATGCATAACC AGGCTCCAAT TCAACGTAGA AAATCAACAC GTATTTACGT
 TGGGAATGTG CCGATTGGCG ATGGTGCCTC CATGCCGTA CAGTCCATGAA CCAATACGCG
 TACGACAGAC GTCGAAGCAA CGGTCAATCA AATCAAGGCG CTGGAACGCG TTGGCGCTGA
 TATCGTCCGT GTATCCGTAC CGACGATGGA CGCGGCAGAA GCGTTCAAAC TCATCAAACA
 CGAGGTTAAC GTGCCGCTGG TGGCTGACAT CCACTTCGAC TATCGCATTG CGCTGAAAGT
 AGCGGAATAC GGCCTCGATT GTCTCGTAT TAACCCCTGGC AATATCGTAA ATGAAGAGCG
 TATTGCGATG TGGGTTGACT GTGCGCGGA TAAAAACATT CCGATCGTA TTGGCGTTAA
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 GCTGGATCT GCCATGCGTC ATGTTGATCA TCTCGATCGC CTGAACTTCG ATCAGTTCAA
 AGTCAGCGTG AAAGCGTCTG ACGTCTTCCT CGCTGTTGAG TCTTATCGTT TGCTGGCAA

ACAGATCGAT CAGCCGTTGC ATCTGGGGAT CACCGAAGCC GGTGGTGCAGC GCAGCGGGGC
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CGTATCGCTG CGGGCCGATC CGGTCGAAGA GATCAAAGTC GGTTTCGATA TTTTGAAATC
GCTGCGTATC CGTTCGCGAG GGATCAACTT CATCGCCTGC CCGACCTGTT CGCGTCAGGA
ATTTGATGTT ATCGGGTACGG TTAACCGCCT GGAGCAACGC CTGGAAGATA TCATCACTCC
GATGGACGTT TCGATTATCG GCTGCGTGGT GAATGGCCCA GGTGAGGCAGC TGGTTCTAC
ACTCGGGCAGC ACCGGCGGCA ACAAGAAAAG CGGCCCTAT GAAGATGGCG TGCGCAAAGA
CCGCTGGAC AACAAACGATA TGATCGACCA GCTGGAAGCA CGCATTGCGT CGAAAGCCAG
TCAGCTGGAC GAAGCGCGTC GAATTGACGT TCAGCAGGTT GAAAATAAG TCGACGAGGA
GAAATTAAAC ATGCAGATCC TGTTGGCCAA CCCGCGTGGT TTTTGTGCCG GGGTAGACCG
CGCTATCAGC ATTGTTGAAA ACGCGCTGGC CATTACGAGC GCACCGATAT ATGTCCTGCA
CGAAGTGGTA CATAACCGCT ATGTTGTCGA TAGCTTGCCT GAGCGTGGGG CTATCTTAT
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TCAGGGCGGT CGTAACGAAG CAAAAAGTCG CGATTTGACG GTGTTGATG CCACCTGTCC
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TCTCATCGGT CACGCCGGGC ACCCGGAAGT GGAAGGGACA ATGGGCCAGT ACAGTAACCC
GGAAGGGGGA ATGTATCTGG TCGAATCGCC GGACGATGTG TGGAAACTGA CGGTCAAAAAA
CGAAGAGAAG CTCTCCTTTA TGACCCAGAC CACGCTGTG TGGGATGACA CGTCTGATGT
GATCGACGCG CTGCGTAAAC GCTTCCGAA ATTGTCGGT CGCGCAGAAG ATGACATCTG
CTACGCCACG ACTAACCGTC AGGAAGCGGT ACGCGCCCTG GCAGAACAGG CGGAAGTTGT
GTTGGTGGTC GGGTCGAAAA ACTCCTCCAA CTCCAACCCTG CTGGCGGAGC TGGCCAGCG
TATGGCAGA CGCGCGTTT TGATTGACGA TCGAAAGAC ATCCAGGAAG AGTGGGTGAA
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TGTGGTGGCA CGTTTGCAGC AGCTGGCGG TGGTGAAGCC ATTCCGCTGG AAGGCCGTGA
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GGTGAGGTGG TTGCTGCGCA AACGGAAAAG CTGACCGTT CGCGCCCGCA TCCACTCTGG
TCGGAACAAG ACCCGGAACA GTGGTGGCAG GCAACTGATC GCGCAATGAA AGCTCTGGC
GATCAGCATT CTCTGCAGGA CGTTAAAGCA TTGGGTATTG CGGGCCAGAT GCACGGAGCA
ACCTTGCTGG ATGCTCAGCA ACGGGTGTAA CGCCCTGCCA TTTTGTGGAA CGACGGGCGC
TGTGCGCAAG AGTGCACCTT GCTGGAAGCG CGAGTTCCGC AATCGGGGT GATTACCGGC
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ACGGGGGAGT TTGCCAGCGA TATGTCGAC GCAGCTGGCA CCATGTGGCT GGATGTCGCA
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GGTATGGCGA CGGTGCCAGT TGTCGCAAGC GGTGGCGACA ATGCAGCTGG TGCAGTTGGT
GTGGGAATGG TTGATGCTAA TCAGGCAATG TTATCGCTGG GGACGTCGGG GGTCTATTTT
GCTGTCAGCG AAGGGTTCTT AAGCAAGCCA GAAAGCGCCG TACATAGCTT TTGCCATGCG
CTACCGAAC GTTGGCATTI AATGTCGTG ATGCTGAGTG CAGCGCTGT TCTGGATTGG
GCCCGAAAT TAACCGGCT GAGCAATGTC CCAGCTTAA TCGCTGCAGC TCAACAGGCT
GATGAAAGTG CCGAGCCAGT TTGGTTCTG CCTTATCTT CCGCGAGCG TACGCCACAC
AATAATCCCC AGGCAGAAGGG GGTGTTCTT GGTGACTC ATCAACATGG CCCCAATGAA
CTGGCGCGAG CAGTGCCTGG AAGCGTGGGT TATGCGCTGG CAGATGGCAT GGATGTCGTG
CATGCCCTGCG GTATTAAACC GCAAAGTGT ACGTTGATT GGGGCGGGGC GCGTAGTGTAG
TACTGGCGTC AGATGCTGGC GGATATCAGC GGTGAGCAGC TCGATTACCG TACGGGGGG
GATGTGGGGC CAGCACTGGG CGCAGCAAGG CTGGCGCAGA TCGCGGGCAA TCCAGAGAAA
TCGCTCATTG AATTGTTGCC GCAACTACCG TTAGAACAGT CGCATCTACC AGATGCGCAG
CGTTATGCG CTTATCAGCC ACGACGAGAA ACGTTCCGTC GCCTCTATCA GCAACTCTG
CCATTAATGG CGTAAAAGCT TGAGGAGAAA TTAACCATGA AGCAACTCAC CATTCTGGGC
TCGACCGGCT CGATTGGTTG CAGCACGCTG GACGTGGTGC GCCATAATCC CGAACACTTC
CGCGTAGTTG CGCTGGTGGC AGGCAAAAT GTCACTCGCA TGGTAGAACAGA GTGCCCTGGAA
TTCTCTCCCC GCTATGCCGT AATGGACGAT GAAGCGAGTG CGAAACTTCT TAAAACGATG
CTACAGCAAC AGGGTAGCCG CACCGAAGTC TTAAGTGGC AACAAAGCCGC TTGCGATATG

GCAGCGCTTG AGGATGTTGA TCAGGTGATG GCAGCCATTG TTGGCGCTGC TGGCTGTTA
CCTACGCTTG CTGCGATCCG CGCGGGTAAA ACCATTTGC TGGCAATAA AGAATCACTG
GTTACCTGCG GACGTCTGTT TATGGACGCC GTAAAGCAGA GCAAAGCGCA ATTGTTACCG
GTCGATAGCG AACATAACGC CATTTCAG AGTTTACCGC AACCTATCCA GCATAATCTG
GGATACGCTG ACCTTGAGCA AAATGGCGTG GTGTCATTT TACTTACCGG GTCTGGTGGC
CCTTCCGTG AGACGCCATT GCGCGATTTG GCAACAATGA CGCCGGATCA AGCCTGCCGT
CATCCGAACT GGTGATGGG GCGTAAAATT TCTGTCGATT CGGCTACCAT GATGAACAAA
GGTCTGGAAT ACATTGAAGC GCGTTGGCTG TTTAACGCCA GCGCCAGCCA GATGGAAGTG
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CGCGTGAACCT CTGGCGTGAA GCCGCTCGAT TTTGCAAAAC TAAGTGCCTT GACATTGCC
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CAGGCAGCGA CGACAGCATT GAATGCCA AAGGAAATCA CCGTTGCTGC TTTCTTGC
CAACAAATCC GCTTACGGA TATGCGTGC TTGAATTAT CCGTACTGGA AAAATGGAT
ATGCGCGAAC CACAATGTGT GGACGATGTG TTATCTGTT ATGCGAACGC GCGTGAAGTC
GCCAGAAAAG AGGTGATGCG TCTCGAAGC TGAGTCGACG AGGAGAAATT AACCATGGCA
ACCACTCATT TGGATGTTG CGCCGTGGT CGGGCGGGCCG GATTGGCCG TCGAATGCAA
ACGGAATGTC CTAAGCAATA TCTCTCAATC GGTAATCAAA CCATTCTGA ACACCTGGTG
CATGCGCTGC TGGCGCATCC CCGGGTAAA CGTGTGTC TTGCCATAAG TCCGGCGAT
AGCCGTTTTG CACAACCTCC TCTGGCGAAT CATCCGAAA TCACCGTTGT AGATGGCGGT
GATGAGCGTG CCGATTCCGT GCTGGCAGGT CTGAAAGCCG CTGGCGACGC GCAGTGGTA
TTGGTGCATG ACGCCGCTCG TCCTTGTGTT CATCAGGATG ACCTCGCGCG ATTGTTGGCG
TTGAGCGAAA CCAGCCGCAC GGGGGGATC CTCGCCGCAC CAGTGCACG TACTATGAAA
CGTGGCGAAC CGGGCAAAAA TGCCATTGCT CATAACGTT ATCGAACCG CTTATGGCAC
GCGCTGACGC CGCAATTTC CCCTCGTGAG CTGTTACATG ACTGTCGAC GCGCGCTCTA
AATGAAGGCG CGACTATTAC CGACGAAGCC TCGGCGCTGG AATATTGCGG ATTCCATCCT
CAGTTGGTCG AAGGCCGTG GGATAACATT AAAGTCACGC GCGCGGAAGA TTTGGCACTG
GCCGAGTTT ACCTCACCCG AACCATCCAT CAGGAGAATA CATAATGCGA ATTGGACACG
GTTTGACGT ACATGCCCTT GGCGGTGAAG GCCAATTAT CATTGGTGGC GTACGCATTC
CTTACGAAAA AGGATTGCTG GCGCATTCTG ATGGCGACGT GGCGCTCCAT GCGTTGACCG
ATGCATTGCT TGGCGCGCG GCGCTGGGG ATATCGGCAA GCTGTTCCCG GATACCGATC
CGGCATTAA AGGTGCCGAT AGCCGCGAGC TGCTACGCGA AGCCTGGCGT CGTATTCAAGG
CGAAGGGTTA TACCCCTGGC AACGTCGATG TCACTATCAT CGCTCAGGCA CCGAAGATGT
TGCGCACAT TCCACAAATG CGCGTGTGTTA TTGCCGAAGA TCTCGGCTGC CATATGGATG
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TTGCCTGTGA AGCGGTGGCG CTACTCATTA AGGCAACAAA ATGACTCGAG GAGGAGAAAT
TAACCATGCG GACACAGTGG CCCTCTCCGG CAAAACTTAA TCTGTTTTA TACATTACCG
GTCAGCGTGC GGATGGTTAC CACACGCTGC AAACGCTGTT TCAGTTCTT GATTACGGCG
ACACCATCAG CATTGAGCTT CGTGACCGATG GGGATATTG TCTGTTAACG CCCGTTGAAG
GCGTGGAAACA TGAAGATAAC CTGATCGTTC GCGCAGCGCG ATTGTTGATG AAAACTGCGG
CAGACAGCGG CGCTTCTCCG ACGGGAAGCG GTGCGAATAT CAGCATTGAC AAGCGTTTGC
CGATGGCGG CGGTCTCGGC GGTGGTTCAT CCAATGCCGC GACGGTCTG GTGGCATTAA
ATCATCTCTG GCAATGCGGG CTAAGCATGG ATGAGCTGGC GGAAATGGGG CTGACGCTGG
GCGCAGATGT TCCCTGTCTT GTTCGGGGC ATGCCGCGTT TGCGAAGGC GTTGGTGA
TACTAACGCC GGTGGATCCG CCAGAGAAGT GGTATCTGGT GGCGCACCC GGTGTAAGTA
TTCCGACTCC GGTGATTTT AAAGATCCTG AACTCCCGCG CAATACGCCA AAAAGGTCAA
TAGAAACGTT GCTAAAATGT GAATTCAAGCA ATGATTGCGA GGTTATCGCA AGAAAACGTT
TTCGCGAGGT TGATGCGGTG CTTTCCTGGC TGTAGAATA CGCCCCGTGCG CGCTGACTG
GGACAGGGC CTGTGTCTT GCTGAATTG ATACAGAGTC TGAAGCCGC CAGGTGCTAG
AGCAAGCCCC GGAATGGCTC AATGGCTTG TGGCGAAAGG CGCTAATCTT TCCCCATTGC
ACAGAGCCAT GCTTTAAGGT ACCCAATTGCG CCCTATAGTG AGTCGTATTA CGCGCGCTCA
CTGGCGCTCG TTTTACAACG TCGTGACTGG GAAAACCTG GCGTTACCA ACTTAATCGC
CTTGCAGCAC ATCCCCCTT CGCCAGCTGG CGTAATAGCG AAGAGGCCG CACCGATCGC
CCTTCCAAC AGTTGCGCAG CCTGAATGGC GAATGGAAAT TGTAAGCGTT AATATTGT
TAAAATCGC GTTAAATTG TGTAAATCA GCTCATTGTT TAACCAATAG GCCGAAATCG

GCAAAATCCC TTATAAAATCA AAAGAATAGA CCGAGATAGG GTTGAGTGTT GTTCCAGTTT
GGAACAAAGAG TCCACTATTA AAGAACGTGG ACTCCAACGT CAAAGGGCGA AAAACCGTCT
ATCAGGGCGA TGGCCCCTA CGTGAACCAT CACCCCTAATC AAGTTTTTG GGGTCGAGGT
GCCGTAAAGC ACTAAATCGG AACCTAAAG GGAGCCCCG ATTTAGAGCT TGACGGGGAA
AGCCGGCGAA CGTGGCGAGA AAGGAAGGGA AGAAAGCGAA AGGAGCGGGC GCTAGGGCGC
TGGCAAGTGT AGCGGTCACG CTGCGCGTAA CCACCAACACC CGCCGCGCTT AATGCGCCGC
TACAGGGCGC GTCAG

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Annex L

DNA and corresponding amino acid sequence of the *ispG* gene (fragment) from *Arabidopsis thaliana*

| | | | | | | | |
|---|-----|-----|-----|-----|-----|-----|-----|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 | |
| >AAGACGGTGAGAAGGAAGACTCGTACTGTTATGGTTGAAATGTCGCCCTTGGAAAGCGAACATCCGATAAGG | | | | | | | |
| K | T | V | R | R | K | T | |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 | |
| I | Q | T | M | T | S | D | |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 | |
| A | G | A | D | I | V | R | |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 | |
| K | L | V | Q | L | N | Y | |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 | 360 |
| G | T | C | G | T | A | G | |
| 370 | 380 | 390 | 400 | 410 | 420 | 430 | |
| I | D | Y | T | E | D | E | |
| 440 | 450 | 460 | 470 | 480 | 490 | 500 | |
| K | C | K | K | Y | G | R | |
| 510 | 520 | 530 | 540 | 550 | 560 | 570 | |
| T | A | T | C | G | G | G | |
| 580 | 590 | 600 | 610 | 620 | 630 | 640 | |
| Y | H | N | F | V | F | S | |
| 650 | 660 | 670 | 680 | 690 | 700 | 710 | 720 |
| A | E | M | Y | V | H | G | |
| 730 | 740 | 750 | 760 | 770 | 780 | 790 | |
| G | R | M | K | S | A | I | |
| 800 | 810 | 820 | 830 | 840 | 850 | 860 | |
| CTGACGGAGCCACCAGAAGAGGGAGATAGATCCCTGCAAGCGATTGGCTAACCTCGGGACAAAAGCTGCCAA | | | | | | | |

L T E P P E E E I D P C R R L A N L G T K A A K

870 880 890 900 910 920 930

CTTCAACAAGCGCTGCACCGTTGAAGAAAAGCATAGGCATTACTTGATTTCAGCGTCGGACGGGTGAT

L Q Q G A A P F E E K H R H Y F D F Q R R T G D

940 950 960 970 980 990 1000

CTACCTGTACAAAAAGAGGGAGAAGAGGGTATTACAGAAATGTCCTCACCGTGATGGTTCTGTTCTGATG

L P V Q K E G E E V D Y R N V L H R D G S V L M

1010 1020 1030 1040 1050 1060 1070 1080

TCGATTTCTGGATCAACTAAAGGCACCTGAACCTCTACAGATCACTCGCCACAAAGCTTGTCTGGGT

S I S L D Q L K A P E L L Y R S L A T K L V V G

1090 1100 1110 1120 1130 1140 1150

ATGCCATTCAAGGATCTGGCAACTGTTGATTCAATCTTATTAAAGAGAGCTACCGCTGTAGATGATCAAGTG

M P F K D L A T V D S I L L R E L P P V D D Q V

1160 1170 1180 1190 1200 1210 1220

GCTCGTTGGCTCTCAAACGGTTGATTGATGTCAGTATGGAGTTATAGCACCTTATCAGAGCAACTAACAA

A R L A L K R L I D V S M G V I A P L S E Q L T

1230 1240 1250 1260 1270 1280 1290

AAGCCATTGCCAATGCCATGGTTCTTGTCAACCTCAAGGAACATCTGGTGGCCTTACAAGCTTCTCCCT

K P L P N A M V L V N L K E L S G G A Y K L L P

1300 1310 1320 1330 1340 1350 1360

GAAGGTACACGCTTGGTTGCTCTCTACAGGCGATGAGCCTTACGAGGAGCTGAAATACTCAAAACATT

E G T R L V V S L R G D E P Y E E L E I L K N I

1370 1380 1390 1400 1410 1420 1430 1440

GATGCTACTATGATTCTCCATGATGTACCTTCACTGAAGACAAAGTTAGCAGAGTACATGCAGCTCGGAGG

D A T M I L H D V P F T E D K V S R V H A A R R

1450 1460 1470 1480 1490 1500 1510

CTATTCGAGTTCTTATCCGAGAATTCAAGTTAACCTTCTGTATTATCGCATCAAACCTCCAACCGGAATC

L F E F L S E N S V N F P V I H R I N F P T G I

1520 1530 1540 1550 1560 1570 1580

CACAGAGACGAATTGGTGATTCATGCAGGGACATATGCTGGAGGCCTTCTTGTTGACTAGGTGATGGC

H R D E L V I H A G T Y A G G L L V D G L G D G

1590 1600 1610 1620 1630 1640 1650

GTAATGCTCGAACCTGACCAAGATTGATTTCTTAGGAATACTTCCCTCAACTTACAAGGATGC

V M L E A P D Q D F D F L R N T S F N L L Q G C

1660 1670 1680 1690 1700 1710 1720

AGAATGCGTAACACTAACGGAATATGTATCGTGCCCGTCTGTGGAAAGAACGCTTTCGACTTGCAAGAA

R M R N T K T E Y V S C P S C G R T L F D L Q E

1730 1740 1750 1760 1770 1780 1790 1800

ATCAGCGCCGAGATCCGAGAAAAGACTTCCCATTACCTGGCTTTCGATCGCAATCATGGGATGCATTGTG

I S A E I R E K T S H L P G V S I A I M G C I V

1810 1820 1830 1840 1850 1860 1870

AATGGACCAGGAGAAATGGCAGATGCTGATTCGGATATGTAGGTGGTCTCCGGAAAAATCGACCTTAT
N G P G E M A D A D F G Y V G G S P G K I D L Y
1880 1890 1900 1910 1920 1930 1940
GTCGGAAAGACGGTGGTGAAGCGTGGGATAGCTATGACGGAGGCAACAGATGCTCTGATCGGTCTGATCAAA
V G K T V V K R G I A M T E A T D A L I G L I K
1950 1960 1970 1980
GAACATGGTCGTTGGGTGACCCGCCGTGGCTGATGAGTAG
E H G R W V D P P V A D E -

Annex M

DNA and corresponding amino acid sequence of the *ispG* (formerly *gcpE*) gene of *Arabidopsis thaliana*

| | |
|---|------|
| ATGGCGACTGGAGTATTGCCAGCTCCGGTTCTGGATCAAGATAACCGGATTGAAAGTC | 60 |
| M A T G V L P A P V S G I K I P D S K V | 20 |
| GGGTTGGTAAAGCATGAATCTTGTGAGAATTGTGATGTTAGGAGTCTAAGATCTGCT | 120 |
| G F G K S M N L V R I C D V R S L R S A | 40 |
| AGGAGAAGAGTTCGGTTATCGGAATTCAAACCAAGGCTCTGATTTAGCTGAGCTTCAA | 180 |
| R R R V S V I R N S N Q G S D L A E L Q | 60 |
| CCTGCATCCGAAGGAAGCCTCTCTTAGTCCAAGACAGAAATATTGTGAATCATTGCAT | 240 |
| P A S E G S P L L V P R Q K Y C E S L H | 80 |
| AAGACGGTGAGAAGGAAGACTCGTACTGTTATGGTTGGAAATGTCGCCCTTGGAAAGCGAA | 300 |
| K T V R R K T R T V M V G N V A L G S E | 100 |
| CATCCGATAAGGATTCAAACGATGACTACTCGGATACAAAGATATTACTGGAACTGTT | 360 |
| H P I R I Q T M T T S D T K D I T G T V | 120 |
| GATGAGGTTATGAGAATAGCGGATAAAGGAGCTGATATTGTAAGGATAACTGTTCAAGGG | 420 |
| D E V M R I A D K G A D I V R I T V Q G | 140 |
| AAGAAAGAGGCGGATGCGTGTGCTTGAATAAAAGATAAACTCGTTAGCCTTAATTACAAT | 480 |
| K K E A D A C F E I K D K L V Q L N Y N | 160 |
| ACACCGCTGGTTGCAGGTATTCACTTGCCCTACTGTAGCCTTACGAGTCGCTGAATGC | 540 |
| T P L V A G I H F A P T V A L R V A E C | 180 |
| TTTGACAAGATCCGTGTCAACCCCGAAATTGGCGGACAGGCGGGCCAGTTGAGACG | 600 |
| F D K I R V N P G N F A D R R A Q F E T | 200 |
| ATAGATTATACAGAAGATGAATATCAGAAAGAACTCCAGCATATCGAGCAGGTCTTCACT | 660 |
| I D Y T E D E Y Q K E L Q H I E Q V F T | 220 |
| CCTTGAGAAATGCAAAAAGTACGGGAGAGCAATGCGTATTGGGACAAATCATGGA | 720 |
| P L V E K C K K Y G R A M R I G T N H G | 240 |
| AGTCTTCTGACCGTATCATGAGCTATTACGGGATTCTCCCGAGGAATGGTTGAATCT | 780 |
| S L S D R I M S Y Y G D S P R G M V E S | 260 |
| GCCTTGAGTTGCAAGAATATGCGGAAATTAGACTATCACAACTTGTGTTCTCAATG | 840 |
| A F E F A R I C R K L D Y H N F V F S M | 280 |
| AAAGCGAGCAACCCAGTGATCATGGTCCAGGCGTACCGTTACTTGTGGCTGAGATGTAT | 900 |
| K A S N P V I M V Q A Y R L L V A E M Y | 300 |
| GTTCATGGATGGATTATCCTTGCAATTGGGAGTTACTGAGGCAGGAGAAGCGAAGAT | 960 |
| V H G W D Y P L H L G V T E A G E G E D | 320 |
| GGACGGATGAAATCTGCGATTGGAATTGGGACGCTTCAGGACGGCTCGGTGACACA | 1020 |

G R M K S A I G I G T L L Q D G L G D T
 ACAAGAGTTCACTGACGGAGCCACCAAGAAGAGGAGATAGATCCCTGCAGGCGATTGGCT 1080
 T R V S L T E P P E E E I D P C R R L A 360
 AACCTCGGGACAAAAGCTGCCAAACTTCAACAAGGCGCTGCACCGTTGAAGAAAAGCAT 1140
 N L G T K A A K L Q Q G A A P F E E K H 380

 AGGCATTACTTGATTTCAGCGTCGGACGGGTGATCTACCTGTACAAAAGAGGGAGAA 1200
 R H Y F D F Q R R T G D L P V Q K E G E 400

 GAGGTTGATTACAGAAATGTCCTCACCGTGATGGTCTGTTCTGATGTCGATTCTCTG 1260
 E V D Y R N V L H R D G S V L M S I S L 420

 GATCAACTAAAGGCACCTGAACCTCTACAGATCACTGCCACAAAGCTGTCGTGGGT 1320
 D Q L K A P E L L Y R S L A T K L V V G 440

 ATGCCATTCAAGGATCTGGCAACTGTTGATTCAATCTTATAAGAGAGCTACCGCCTGTA 1380
 M P F K D L A T V D S I L L R E L P P V 460

 GATGATCAAGTGGCTCGTTGGCTCTCAAACGGTGATTGATGTCAGTATGGGAGTTATA 1440
 D D Q V A R L A L K R L I D V S M G V I 480

 GCACCTTATCAGAGCAACTAACAAAGCCATTGCCAATGCCATGGTCTTGTCAACCTC 1500
 A P L S E Q L T K P L P N A M V L V N L 500

 AAGGAACTATCTGGTGGCGCTTACAAGCTCTCCCTGAAGGTACACGCTTGGTTGTCTCT 1560
 K E L S G G A Y K L L P E G T R L V V S 520

 CTACGAGGCATGAGCCTTACGAGGAGCTGAAATACTCAAAACATTGATGCTACTATG 1620
 L R G D E P Y E E L E I L K N I D A T M 540

 ATTCTCCATGATGTACCTTCACTGAAGACAAAGTTAGCAGAGTACATGCAGCTGGAGG 1680
 I L H D V P F T E D K V S R V H A A R R 560

 CTATTGAGTTCTTATCCGAGAATTCACTTAACCTTCTGTTATTCAATCGCATAAACTTC 1740
 L F E F L S E N S V N F P V I H R I N F 580

 CCAACCGGAATCCACAGAGACGAATTGGTGATTCAATGCAGGGACATATGCTGGAGGCCTT 1800
 P T G I H R D E L V I H A G T Y A G G L 600

 CTTGTGGATGGACTAGGTGATGGCGTAATGCTCGAAGCACCTGACCAAGATTTGATTTT 1860
 L V D G L G D G V M L E A P D Q D F D F 620

 CTTAGGAATACTCCTCAACTTATTACAAGGATGCAGAATGCGTAACACTAACAGACGGAA 1920
 L R N T S F N L L Q G C R M R N T K T E 640

 TATGTATCGTGCCGTCTGTGGAAAGAACGCTTTCGACTTGCAAGAAATCAGGCCGAG 1980
 Y V S C P S C G R T L F D L Q E I S A E 660

 ATCCGAGAAAAGACTTCCCATTACCTGGCGTTCGATCGCAATCATGGGATGCATTGTG 2040
 I R E K T S H L P G V S I A I M G C I V 680

 AATGGACCAGGAGAAATGGCAGATGCTGATTCGGATATGTAGGTGGTTCTCCGGAAAA 2100

N G P G E M A D A D F G Y V G G S P G K 700

ATCGACCTTATGTCGGAAAGACGGTGGTGAAGCGTGGGATAGCTATGACGGAGGCAACA 2160
I D L Y V G K T V V K R G I A M T E A T 720

GATGCTCTGATCGGTCTGATCAAAGAACATGGTCGTTGGGTCGACCCGCCGTGGCTGAT 2220
D A L I G L I K E H G R W V D P P V A D 740

GAGTAG 2226

E - 741

Annex N

cDNA sequence of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (lspH)
from *Arabidopsis thaliana*

| | | | | |
|---|-----------------------------|-------------------------|------------------------------|------|
| ATGGCTGTTGCGCTCCAATT | CAGCCGATTATGCGTCGACGGGATACT | TTCGTGC | CGGGAGAATCATCTCTCT | 72 |
| M A V A L Q F S R L C V R P D T F V R E N H L S | | | | 24 |
| GGATCCGGATCTCTCCG | CCGCCGGAAAGCTTATCAGTCCGGT | GCTCGT | GGCGATGAGAACGCTCCTTCG | 144 |
| G S G S L R R R K A L S V R C S S G D E N A P S | | | | 48 |
| CCATCGGTGGT | GATGGACTCCGATT | CGACGCCAAGGT | GTTCCGTAAGAAC | 216 |
| P S V V M D S D F D A K V F R K N L T R S D N Y | | | | 72 |
| AATCGTAAAGGGTTCGGT | CATAAGGAGGGAGACACTCAAGCT | CATGAATCGAGAGTACACCAGT | GATATATTG | 288 |
| N R K G F G H K E E T L K L M N R E Y T S D I L | | | | 96 |
| GAGACACTGAAAACAA | ATGGGTATACTTATTCTGGGAGATGTT | ACTGTGAAACTCGCTAAAGC | CATATGGT | 360 |
| E T L K T N G Y T Y S W G D V T V K L A K A Y G | | | | 120 |
| TTTGCTGGGTGTTGAGCGT | GCTGTTCAAGATTGCAATATG | AGCACGAAAGCAGTTCCAGAGG | GAGGGCTT | 432 |
| F C W G V E R A V Q I A Y E A R K Q F P E E R L | | | | 144 |
| TGGATTACTAACGAAATCATT | CATAACCCGACCGTC | AAATAAGAGGTTGGAAGA | ATATGGATGTTAAATTATT | 504 |
| W I T N E I I H N P T V N K R L E D M D V K I I | | | | 168 |
| CCGGTTGAGGATTCAAAGAA | ACAGTTGATGTA | GTAGAGAAAGATGATG | TGGTTATCCTCCTGC | 576 |
| P V E D S K K Q F D V V E K D D V V I L P A F G | | | | 192 |
| GCTGGTGGT | GACGAGATGTA | TGTTCTTAATGATA | AAAAGTGCAAATTGTTGACACGACTTGT | 648 |
| A G V D E M Y V L N D K K V Q I V D T T C P W V | | | | 216 |
| ACAAAGGTCTGGAACACGGT | TGAGAAGCACAAGAAGGGGA | ATACACATCAGTAATCCATGG | AAATATAAT | 720 |
| T K V W N T V E K H K K G E Y T S V I H G K Y N | | | | 240 |
| CATGAAGAGACGATTGCA | ACTGCGTCTTGCAGGAA | AGTACATCATTGTA | AAAGAACATGAAAGAGGCAA | 792 |
| H E E T I A T A S F A G K Y I I V K N M K E A N | | | | 264 |
| TACGTTGATTACATT | TCGGTGGCAATACGATGG | ATCTAGCTCCACAAAAGAGGAGT | CATGGAGAAA | 864 |
| Y V C D Y I L G G Q Y D G S S S T K E E F M E K | | | | 288 |
| TTCAAATACGCAATT | CGAAGGGTTCGATCCC | GACAATGACCTTGTC | AAAGTTGGTATTGCAAACCAAACA | 936 |
| F K Y A I S K G F D P D N D L V K V G I A N Q T | | | | 312 |
| ACGATGCTAAAGGGAGAA | ACAGAGGAGATAGGAAGA | ATTACTCGAGACAACA | ATGATGCGCAAGTATGGAGTG | 1008 |
| T M L K G E T E E I G R L L E T T M M M R K Y G V | | | | 336 |
| GAAAATGTAAGCGGACATT | CATCAGCTTCAACACA | ATATGCGACGCTACTCAAGAGCG | ACAAGACGCAATC | 1080 |
| E N V S G H F I S F N T I C D A T Q E R Q D A I | | | | 360 |
| TATGAGCTAGTGGAAAGAGA | AGATTGACCTCATGCTAGT | GGTGGCGGATGGA | ATTCAAGTAACACCTCTCAC | 1152 |
| Y E L V E E K I D L M L V V G G W N S S N T S H | | | | 384 |
| CTTCAGGAAATCTCAGAGGCACGGGA | ATCCCACATTACTGG | GATCGATAGTGAGAA | ACGGATAGGACCTGGG | 1224 |
| L Q E I S E A R G I P S Y W I D S E K R I G P G | | | | 408 |
| AATAAAATAGCTTATAAGCT | CCACTATGGAGAAC | TTGGTCGAGAAGGAA | ACTTCTCCAAAGGGACCAATA | 1296 |
| N K I A Y K L H Y G E L V E K E N F L P K G P I | | | | 432 |
| ACAATCGGTGTGACATCAGGT | GATCAACCCCGATAAG | TGCGTGGAAAGATG | CTTGGTGAAGGTGTTGAC | 1368 |
| T I G V T S G A S T P D K V V E D A L V K V F D | | | | 456 |
| ATTAAACGTGAAGAGTTATTG | CAGCTGGCTTGA | | | 1398 |
| I K R E E L L Q L A - | | | | 466 |